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THE MODULATION OF VOLTAGE-DEPENDENT CALCIUM CHANNELS IN UMR-106 CELLS BY 24,25$(OH)_2$ VITAMIN D_3

BY

BING LI

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

DEPARTMENT OF PHYSIOLOGY

EDMONTON, ALBERTA

SPRING, 1995

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DEDICATION

To

My lovely wife Hua Li, my son Xiaorui Li,

My parents, Min Li and Yinzhu Hong,

My parents in law, Xingqi Li, and Zheng Wu,

For their love, understanding and support.

THE MODULATION OF VOLTAGE-DEPENDENT CALCIUM CHANNELS BY 24,25(OH)$_2$ VITAMIN D$_3$ IN UMR-106 CELLS

ABSTRACT

The genomic effects of vitamin D$_3$ and its metabolites involve activation of intracellular receptors, gene transcription and protein synthesis. Vitamin D$_3$ and its metabolites can also quickly affect intracellular calcium concentration through their nongenomic actions. The nongenomic effect of 24,25(OH)$_2$ vitamin D$_3$ (24,25D$_3$), a major metabolite, remains controversial. In this thesis, the nongenomic effect of 24,25D$_3$ on voltage-dependent calcium channels was studied in UMR-106 cells, an osteogenic sarcoma cell line. Two types of voltage dependent calcium channels were expressed in this cell line with different patterns. The L type channels were expressed in cells from all passages while the T type channels were mainly expressed in cells from high passages. The T channels had the highest current density after being subcultured and the current density declined as the culture time was prolonged. The current density of the L channels showed a gradual increase during subculture.

24,25D$_3$ had a biphasic effect on the L type calcium channel current. At concentrations below 10^{-7}M, it increased the L type calcium channel current. At concentrations higher than 10^{-7}M, it inhibited the current. The biphasic effect of 24,25D$_3$ was reversible, suggesting that it may be mediated by a membrane-associated process.

The biphasic effect of 24,25D$_3$ on the L type calcium channel current was

mediated by cyclic AMP/protein kinase A and protein kinase C signalling pathways with the involvement of G proteins. The effect of low concentrations of $24,25D_3$ on the L type calcium channel current was mediated by the cyclic AMP/protein PKA pathway. At high concentrations of $24,25D_3$, both cyclic AMP/protein kinase A and protein kinase C pathways were involved with protein kinase C playing a dominant role. This resulted in an inhibitory effect of $24,25D_3$ on the L type calcium channel current.

This study demonstrates for the first time that the expression of T and L type calcium channels in UMR-106 cells depends on culture conditions. It is also the first demonstration of a nongenomic effect of $24,25D_3$ and the involvement of intracellular second messenger systems in the modulation of voltage-dependent calcium channel currents in UMR-106 cells.

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LIST OF ABBREVIATIONS

$1,25D_3$	$1,25(OH)_2$ vitamin D_3
$24,25D_3$	$24,25(OH)_2$ vitamin D_3
$25D_3$	25(OH) vitamin D_3
ATP	adenosine triphosphate
AC	adenylate cyclase
ACh	acetylcholine
4-AP	4-aminopyridine
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
cAMP	adenosine 3',5'-cyclicmonophosphate
cGMP	guanosine 3',5'-cyclicmonophosphate
CaBP	calcium binding protein
CTX	cholera toxin
DAG	diacylglycerol
DBP	vitamin D binding protein
DMEM	Dulbecco's modified Eagle's medium
DRG	dorsal root ganglion
DHP	dihydropyridine
DMSO	dimethylsulfoxide
EGTA	ethyleneglycol-bis-(-aminoethylether) N,N,N',N',-tetraacetic acid

FBS	fetal bovine serum
FTX	funnel web spider toxin
GDP βS	guanosine-5'-O-(2-thiodiphosphate)
G protein	guanine nucleotide binding protein
GTP γS	guanosine-5'-O-(3-thiotriphosphate)
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IP_3	inositol 1,4,5-trisphosphate
I-V relationship	current-voltage relationship
L-type Ca^{2+} channel	long-lasting calcium channel
OAG	1-oleoyl-2-acetyl-rac-glycerol
P^+	phosphate
PI	phosphatidylinositol
PIP_2	phosphatidylinositol 4,5-bisphosphate,
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PMA	4β-phorbol 12-myristate 13-acetate
PTH	parathyroid hormone
PTX	pertussis toxin

Rp-cAMPs	adenosine 3',5'-cyclicmonophosphothioate Rp-isomer
SR	sarcoplasmic reticulum
T-type Ca^{2+} channel	transient calcium channel
TTX	tetrodotoxin
VDCC	voltage dependent calcium channel
VSMC	vascular smooth muscle cell

Chapter I

Introduction and Literature Review

1.1. The discovery and history of vitamin D and its metabolites

The importance of vitamin D in maintaining the normal physiological function of the body has been recognized for a long time. In 1919, Sir Edward Mellanby showed that a deficiency of a fat-soluble substance in the diet resulted in the disease rickets (Mellanby, 1919). Since this disease could be cured using cod liver oil, he concluded that the anti-rachitic factor was of dietary origin. Later, McCollum and his co-workers clearly demonstrated that this anti-rachitic activity of cod liver oil was the result of a factor which he named vitamin D (McCollum, 1922). Another important observation was that of Huldschinsky (1919), who demonstrated that rickets in children could be cured not only by cod liver oil but also by ultraviolet light. Steenbock and Black (1924) showed that ultraviolet irradiation converts an inactive substance to the anti-rachitic factor and this led to their isolation and identification of vitamin D_2 and the determination of its structure. After that, research concerning vitamin D entered a new stage with the development of more sophisticated techniques and equipment. In the late 1930's, vitamin D_3 was synthesized and thus, its physiological action could be studied. During this period, vitamin D_3 was shown to stimulate intestinal calcium absorption as well as the mobilization of calcium from bone (summarized by Deluca, 1974). Several metabolites of vitamin D were discovered in the 1960's as the result of the chemical synthesis of

radioactive vitamin D with high specific activity. In 1971, DeLuca and co-workers isolated and identified 1α,25(OH)$_2$ vitamin D$_3$ (1,25D$_3$) and later provided conclusive evidence by chemical synthesis that the active form of vitamin D$_3$ is 1,25D$_3$ (DeLuca, et al., 1990). It is at this point that the modern era of vitamin D investigation began. The following is a review of the modern concepts of the vitamin D system. The focus will be on two major metabolites of vitamin D$_3$: 1,25D$_3$ and 24,25(OH)$_2$ vitamin D$_3$ (24,25D$_3$).

1.2. The molecular structure of vitamin D

Based on their chemical structure, vitamin D and its metabolites are seco-steroids. A seco-steroid is one in which either the A,B,C, or D ring of the typical cyclopentanoperhydrophenanthrene ring of cholesterol or 7-dehydrocholesterol backbone is broken (see Fig.I-1). In the case of vitamin D, it is the 9-10 carbon bond of ring B which is broken. If the side chain is equivalent to that of cholesterol, the compound is vitamin D$_3$; if the side chain is that of ergosterol, it is vitamin D$_2$. At the present time, 37 metabolites of vitamin D$_3$ have been isolated and chemically characterized (Henry and Norman, 1992).

1.3. Photoproduction of vitamin D$_3$ and synthesis of 1,25 D$_3$ and 24,25 D$_3$

Vitamin D$_3$ can either be obtained from dietary sources or biosynthesized by the skin. The precursor of vitamin D$_3$ is 7-dehydrocholesterol (provitamin D$_3$). In humans, one half of the 7-dehydrocholesterol is found in the epidermis and the other half in the

dermis. When the skin is exposed to sunlight, the ultraviolet B portion (290-315 nM) of the solar spectrum converts the provitamin D_3 photochemically to previtamin D_3. Once formed, previtamin D_3 immediately begins to equilibrate thermally to vitamin D_3. The vitamin D_3 then binds to the vitamin D binding protein and is released into the circulation and transported to the liver. In the liver, vitamin D_3 undergoes 25-hydroxylation by 25-hydroxylase and 25-hydroxyvitamin D_3 ($25D_3$) appears in the circulation. The $25D_3$ has bioactive effects. However, at physiological concentrations, its action appears to be very weak and it requires further metabolism in the kidney which contains several $25D_3$-hydroxylases. The most important of these are $25D_3$-1α-hydroxylase and $25D_3$-24-hydroxylase. These two key enzymes are controlled by a number of ionic and endocrine factors including stimulation by parathyroid hormone (PTH) and reduced plasma calcium or inhibition by $1,25D_3$ in a classic negative feedback loop. When the demand for calcium is increased, 1α-hydroxylase is activated by PTH and $25D_3$ is converted to $1,25D_3$, the most potent bioactive form of vitamin D_3, which is responsible for most of the physiological actions of vitamin D_3. When serum calcium levels and $1,25D_3$ concentrations are high, 24-hydroxylase is activated and converts $25D_3$ to $24,25D_3$ which was thought to be biologically inactive (Henry and Norman, 1992; Holick, 1989). Fig.I-2 shows the pathways of the biosynthesis of $1,25D_3$ and $24,25D_3$.

1.4. The actions of Vitamin D_3 metabolites

1.4.1. The genomic actions of vitamin D_3 metabolites

Traditionally, vitamin D metabolites were thought to exert their principal biological action by the activation of specific intracellular receptors (reviewed by Civitelli and Avioli, 1994). After vitamin D_3 binds to its intracellular receptor, the vitamin D-receptor complex initiates gene transcription and synthesis of functional proteins, such as enzymes and calcium binding proteins. The functional proteins in turn induce the biological action. Since this classical pathway of signal transduction utilized by vitamin D involves gene transcription and protein synthesis, it requires a long time for processing, *i.e.*, hours or days (Wehling, 1994). Vitamin D metabolites play a very important role in maintaining calcium homeostasis by their classic genomic actions on three important target organs: bone, kidney and intestine.

1.4.1.1 Action on bone

It is well established that vitamin D and its metabolites stimulate bone resorption and mobilize calcium from bone to the blood. $1,25D_3$, the most potent hormonal form of the vitamin D metabolites, plays a major role in this process. Receptor-binding studies have demonstrated that the osteoblast rather than the osteoclast is the target cell in bone for $1,25D_3$. Autoradiographic studies in 18- to 20-day-old rat fetuses injected with ^3H-$1,25D_3$ have shown the presence of radioactivity in the nuclei of osteoblasts and osteoprogenitor cells. In contrast, no labelling has been observed in mature

multinucleared osteoclasts (Narbaitz *et al.*, 1983). Furthermore, $1,25D_3$ does not increase the motility of isolated mature osteoclasts. However, the resorption of cortical bone slices by osteoclasts is activated when osteoclasts are co-cultured with osteoblasts. An as yet uncharacterized osteoclast-stimulating factor that promotes bone resorption is released when osteoblasts are treated with $1,25D_3$ (McSheehy *et al.*, 1987). These results, obtained from isolated cell populations, are consistent with the model established by Rodan and Martin (1981) in which $1,25D_3$ initially affects bone resorption by a direct action on the osteoblast which then activates the osteoclast via a local hormonal mediator.

Another way in which vitamin D_3 metabolites produce their effects on bone is to increase the numbers of osteoclasts. Several experiments have yielded evidence which indicates that $1,25D_3$ promotes the differentiation of monocytic cells such as normal mouse alveolar macrophages and HL-60 promyocytic leukaemic cells, as well as primate marrow mononuclear cells with osteoclast-like characteristics including multinucleation and tartrate-resistant acid phosphatase activity (Abe *et al.*, 1983; Bar-Shavit *et al.*, 1983; Roodman *et al.*, 1983). $1,25D_3$ also induces characteristic changes in osteoclast number, size, nuclear area, ruffled border and clear zone (Holtrop and Raisz, 1979). Other metabolites of vitamin D_3 ($24,25D_3$ and $25D_3$) exhibit similar but less potent bone resorption effects (Walter, 1992). Therefore, $1,25D_3$, as well as other vitamin D metabolites, indirectly activates osteoclasts and promotes bone resorption through its action on osteoblasts.

The $1,25D_3$ has other actions which affect bone resorption and differentiation of

bone cells. The synthesis of collagen is inhibited by $1,25D_3$ in several systems *in vitro*, including mouse and rat calvaria and cells derived from these bone tissues. The inhibitory effects of $1,25D_3$ on collagen synthesis paralleled that for bone resorption (Raisz *et al.*, 1978).

Several other proteins synthesized by bone cells appear to be regulated by 1,25 D_3. Osteocalcin, an important protein in forming the bone matrix, is stimulated after bone cells are treated for 24 hours with $1,25D_3$ or other metabolites of vitamin D_3 (Holtrop and Raisz, 1979). It has been reported that alkaline phosphatase activity is influenced by $1,25D_3$ and a number of other vitamin D metabolites both in rodent and human osteoblasts. Alkaline phosphatase activity in fetal rat calvaria is significantly inhibited after a 24 hour culture with $1,25D_3$ (Analis *et al.*, 1983). In contrast, $1,25D_3$ stimulated the activity of alkaline phosphatase in human osteosarcoma cells (Mulkins *et al.*, 1983). Observations over the last decade indicate that the effects of vitamin D_3 and its metabolites on the activity of alkaline phosphatase are related to cell type (Kurihara *et al.*, 1986).

Taking the above observations together, it appears that the vitamin D_3 family, by their genomic actions, indirectly activate osteoclasts by increasing their number, size and activity, inhibit the activity of osteoblasts and regulate the synthesis of proteins and the activity of enzymes required for bone mineralization. Through these actions, vitamin D_3 promotes bone resorption and releases calcium from bone tissue into the blood when the blood calcium concentration is decreased.

1.4.1.2. Action on kidney

The kidney is a principal target tissue of vitamin D metabolites. Regulation of calcium and phosphate reabsorption from the urine by these metabolites plays a very important role in calcium homeostasis (Holic, 1989). The kidneys of a typical adult male transfer about 11,000 mg (275 nmoles) of calcium per day from the plasma into the glomerular filtrate. However, only 0.5-1.0% (100-200 mg) of filtered calcium is excreted daily in the urine. The concentration of phosphorus in the blood is not as strictly regulated as that of calcium. The inorganic phosphate in the blood is also very efficiently filtered in the glomeruli. In a typical day, approximately 6000-10,000 mg of phosphate are transferred from the blood to the glomerular filtrate. Of this amount the renal tubule reabsorbs 80-90%. A normal adult male loses 700-1500 mg of phosphate daily in the urine (Norman, 1979).

Attention has focused on the possible role of vitamin D in the renal reabsorption of phosphorus and calcium. As early as 1964, the administration of vitamin D to intact animals was shown to suppress parathyroid hormone secretion and this resulted in the suppression of parathyroid hormone-mediated phosphaturia, indicating that vitamin D had an indirect effect on phosphorus (Harrison and Harrison, 1971). Following the discovery of the various vitamin D metabolites, much effort was made to demonstrate the effects of vitamin D on calcium reabsorption in the kidney. In 1972, Taylor and Wasserman reported the presence of a vitamin D-dependent calcium-binding protein (CaBP) in the chick kidney. Chicks which were fed a vitamin D-deficient diet became progressively more rachitic with a concomitant fall in the serum calcium concentration and the

concentration of renal CaBP. This suggests that the CaBP may participate in the calcium reabsorption in the kidney and may be modulated by vitamin D_3. However, as is the case for the intestinal CaBP, no conclusive studies have yet been presented showing a functional involvement of the renal CaBP in the renal metabolism of calcium (Taylor and Wasserman, 1972). In support of this hypothesis, is the observation that in vitamin D-deficient parathyroid ctomized rats stabilized on a diet containing either 0.1% or 0.02% phosphorus, a dose of 0.65 - 1.3 nmoles $1,25D_3$ resulted in a significant lowering of the fraction of filtered calcium excreted in the urine (Steele *et al.*, 1975; Puschett *et al.*, 1975). Nevertheless, while the effects of vitamin D on the kidney have been illustrated, the mechanism by which vitamin D modulates the reabsorption of calcium in the kidney still remains unclear (Norman, 1979).

1.4.1.3. Action on the intestine

Another important organ where vitamin D_3 has a profound effect in maintaining calcium and phosphorous homeostasis is the small intestine, especially the duodenum. Although the whole intestine is involved in absorbing calcium and phosphorous, the majority of the calcium transport activity is localized in the duodenum. It has been shown that $1,25D_3$ directly affects: 1) the entry of calcium through plasma membrane into intestinal absorptive cells; 2) the movement of calcium through the cytoplasm; 3) the transfer of calcium across the basolateral membrane into the circulation (Holick, 1989). In the vitamin D-deficient rat, vitamin D and its metabolites have been shown to increase the concentrations of several proteins which are involved in absorbing calcium in the

small intestine, for example, CaBP. There is a close association between the amount of CaBP and intestinal calcium absorption. However, it has also been shown that, when the calcium absorption decreased to the vitamin D-deficient level, the synthesis of CaBP induced by vitamin D remained high (Lawson, 1984; Holick, 1989). This suggests that the synthesis of CaBP induced by $1,25D_3$ is not the sole control of calcium transport in intestine. Bikle *et al.*, (1984) demonstrated that, $1,25D_3$ increased phosphatidylethanolamine and phosphatidylcholine content in intestinal cells of the vitamin D-deficient chick. This alteration appears to be correlated with calcium permeability of the brush-border membrane vesicles. The lack of essential fatty acids in rats could also inhibit the effects of $1,25D_3$ on the calcium absorption from intestine (Putkey *et al.*, 1982). Thus, the changes in membrane permeability and fluidity induced by vitamin D and its metabolites may be one mechanism by which vitamin D_3 enhances the efficiency of the intestinal calcium absorption (Rasmussen *et al.*, 1982; Pukey *et al.*, 1982; Brasitus *et al.*, 1986). In general, it is accepted that vitamin D and its metabolites enhance intestinal calcium absorption by multiple mechanisms.

1.4.1.4. Action on other systems

The specific nuclear receptors for vitamin D_3 have been found in many tissues that are not considered to be primary sites of action of vitamin D, such as parathyroid cells (Hughes and Haussler, 1978; Brumbaugh *et al.*, 1975), pancreas (Clark *et al.*, 1987), vascular smooth cells (Merke *et al.*, 1987), nerve cells (Stumpf *et al.*,1979; 1987), leukocytes (Provvedini *et al.*,1983), peripheral blood mononuclear cells (Bhalla *et al.*,

1983) and the reproductive system (Halloran, 1989). In these tissues, vitamin D_3 and its metabolites are not involved in the regulation of calcium homeostasis. Therefore, they appear to modulate growth and differentiation, for example, in myeloid cell differentiation (Suda, 1989), human cancer cell replication (Eisman *et al.*, 1989), and T cell differentiation (Manolagas *et al.*, 1989). Since the effects of vitamin D_3 on these tissues are not the focus of this study, they are not discussed in detail in this reivew.

1.4.2. The nongenomic effects of vitamin D_3 and its metabolites: the actions on intracellular signalling systems

As discussed previously, the genomic actions of vitamin D_3 and its metabolites on target tissues represent an important control mechanism that maintains plasma calcium at an appropriate concentration. The genomic effects of vitamin D_3 need hours or days to develop since gene transcription, mRNA and protein synthesis are involved (Wehling, 1994).

During the last decade, a new aspect of the actions of vitamin D_3 has been established. It has become apparent that vitamin D_3 and its metabolites as well as other steroids, can generate biological effects on target cells via nongenomic pathways. These nongenomic effects require only seconds or minutes to develop and are insensitive to the inhibitors of transcription or protein synthesis, such as actinomycin D and cycloheximide (Cancela *et al.*, 1988; Baran *et al.*, 1988; 1992; Norman *et al.*, 1991; 1993; Wehling, 1994).

In 1984, it was reported that $1,25D_3$ could stimulate rapid (within 2-4 min)

calcium transport in vascular perfused chick duodenal loops. This rapid $1,25D_3$-mediated transport of calcium was termed transcaltachia and was independent of the gene transcription effects of $1,25D_3$ (Norman *et al.*, 1984). Following this pioneering work, many rapid nongenomic effects induced by vitamin D_3 metabolites have been observed in both classical and nonclassical target tissues. Among these nongenomic effects of vitamin D_3, the modulation of intracellular second messenger systems by vitamin D_3 metabolites has received much attention.

1.4.2.1. Effect on intracellular calcium concentration ($[Ca^{2+}]_i$)

$[Ca^{2+}]_i$ is well recognized as an intracellular second messenger. The alteration of $[Ca^{2+}]_i$ involves the mobilization of intracellular calcium stores and the activation of calcium channels (either voltage-dependent or receptor-gated calcium channels) (Tsein and Tsein, 1990). Vitamin D_3 metabolites have been reported to induce a rapid and transient increase in $[Ca^{2+}]_i$ in many tissues.

In 1984, it was reported that calcium transport across the intestinal membrane could be enhanced within 15 min of vascular perfusion with $1,25D_3$ (Norman *et al.*, 1984). This increase was correlated with an increase in $[Ca^{2+}]_i$. It was further demonstrated that this rapid hormone-stimulated intestinal calcium transport resulted from the opening of L type voltage-dependent calcium channels (VDCC) since this increase in calcium transport could be mimicked by Bay K 8644 or blocked by nifedipine. Other metabolites of vitamin D_3, such as $24,25D_3$, were also shown to produce similar effects but with a lesser potency (Norman *et al.*, 1984, 1987, 1992, 1994). Following these

reports, similar results were reported in different cell types. It has been confirmed that vitamin D_3 increases $[Ca^{2+}]_i$ in rat enterocytes (DeLuca *et al.*, 1989), colonic epithelial cells (Wali *et al.*,1990), amphibian kidney tubules (Edelman *et al.*, 1988), lymphoid cells (Desai *et al.*, 1986), mammary tissue (Mezzetti *et al.*, 1988), hepatocytes (Baran and Kelly 1988), osteoblasts (Lieberherr *et al.*,1987; Caffrey and Farach-Carson, 1989; Civitelli *et al.*, 1990) and cultured cardiac fibroblasts (Wehling, 1994).

All these effects on $[Ca^{2+}]_i$ induced by vitamin D_3 metabolites take place rapidly and transiently. The increase in the $[Ca^{2+}]_i$ induced by vitamin D_3 metabolites appears to involve the inward movement of calcium through calcium channels and the release of intracellular calcium.

Using the patch clamp technique, Caffrey and Farach-Carson (1989) demonstrated that, at physiological concentrations, $1,25D_3$ increased the L type calcium current in ROS 17/2.8 cells and shifted the activation threshold to a more negative potential. The $24,25D_3$ and $25D_3$ exhibited similar but less potent stimulatory effects (Caffrey and Frarch-Carson. 1989). The $1,25D_3$ has also been shown to increase the L type calcium current in smooth muscle cells from rat tail arteries (Shan *et al.*, 1993) and in rat cardiac myocytes (de Boland, 1993).

Using the intracellular calcium indicator Fura-2, it has been demonstrated that, in addition to their effects on calcium channels, vitamin D_3 metabolites also affect calcium release from intracellular stores. For example, the application of $1,25D_3$ and $25D_3$ induced a rapid increase in intracellular calcium in ROS 17/2.8 cells. This increase was composed of both calcium influx and release of intracellular calcium, since this

increase in $[Ca^{2+}]_i$ could only be partially blocked by nifedipine. In contrast, $24,25D_3$ only induced calcium release from the sarcoplasmic reticulum (SR) in ROS 17/2.8 cells (Lieberherr *et al.*, 1987). The source of increase in intracellular calcium by some vitamin D_3 metabolites seems dependent on the concentration. It was reported that, at concentrations exceeding 0.1uM, $1,25D_3$ induced an influx of calcium and the release of Ca^{2+} from intracellular stores, whereas at concentrations lower than 0.01uM, $1,25D_3$ only caused an influx of extracellular calcium (Lieberherr *et al.*, 1987).

In contrast to $1,25D_3$, the effect of $24,25D_3$ on the intracellular calcium is controversial. As described above, $24,25D_3$ was demonstrated to release calcium from SR in ROS 17/2.8 cells. In contrast to this observation by Liberherr and co-workers, Civitelli *et al.*, (1990) found no effect of $24,25D_3$ on either calcium influx or intracellular calcium release in the same cell line.

In summary, vitamin D_3 metabolites produce significant and rapid effects on $[Ca^{2+}]_i$ in many cells by modulating voltage-dependent calcium channels and by activation of intracellular calcium stores. However, the specific mechanisms underlying these effects remains controversial.

1.4.2.2. Effects on the cAMP/PKA signal pathway

The cAMP/PKA signal transduction pathway is a very important second messenger system which modulates many cellular functions by coupling extracellular signals to intracellular responses. When a ligand binds to the appropriate receptor, it stimulates cAMP accumulation and activates the cAMP-dependent protein kinase (PKA).

The activated PKA can then phosphorylate many target proteins, (*i.e*, channel proteins), changing the functions of these proteins, and causing biological effects such as the opening and/or changing the state of ion channels (Chang *et al.*, 1991), the induction hormone release (Gwosdow *et al.*, 1993), and the relaxation of smooth muscle cells (Zheng *et al.*, 1989).

Vitamin D_3 and its metabolites have been shown to modulate the cAMP/PKA pathway in some cells, including muscle cells (Bellido *et al.*, 1993), intestinal cells (de Boland *et al.*, 1990), myoblasts (Drittanti *et al.*, 1993), bone, and kidney cells from rat (Gordeladze and Gautvik, 1986). The effects of vitamin D_3 metabolites on the cAMP/PKA pathway in bone cells have been observed and are the subject of debate. It has been shown that vitamin D_3 metabolites do not affect intracellular cAMP accumulation in bone, but they can change the sensitivity of cAMP and PKA to other agonists. Catherwood (1985) reported that treatment of ROS 17/2.8 cells with $1,25D_3$ attenuated the cAMP response to subsequent stimulation by PTH (Catherwood, 1985). Similar results were reported by Rao and Wylie (1993) and Martin (1985). $24,25D_3$ has also been reported to inhibit PTH-stimulated adenylate cyclase activity in osteoblasts (Klem *et al.*, 1990.), rat calvaria (Gordeladze and Gautvik, 1986) and in iliac crest biopsies from uraemic patients (Mortensen *et al.*, 1993). The above evidence suggests that vitamin D_3 and its metabolites may affect cAMP/PKA signal pathway in an indirect manner. However, it should be pointed out that these results of vitamin D_3 and its metabolites on cAMP accumulation in bone cells were obtained from experiments in which the cells were treated with vitamin D_3 and its metabolites for 24 to 48 hours.

Under these experimental conditions, the effect of vitamin D_3 and its metabolites on the cAMP/PKA pathway may involve genomic actions. The acute effect (nongenomic action) of vitamin D_3 and its metabolites on this signal transduction system has not been well characterized. Thus, whether vitamin D_3 metabolites activate or modulate the cAMP/PKA pathway remains unclear.

1.4.2.3. Effects of vitamin D_3 on the phospholipase C (PLC)/Protein Kinase C (PKC) pathways

Similar to the cAMP/PKA pathway, PLC/PKC forms another important signal pathway which transforms the outside signal induced by ligand-receptor coupling into a cellular response. It has been well documented that the binding of ligands to their membrane receptors on target cells activates G proteins which, in turn, may activate PLC (Shearman *et al.*, 1989). Activated PLC acts on the phosphoinositides in the membrane and produces inositol 1,4,5, triphosphate (IP_3), a potent stimulator of intracellular calcium release, and diacylglycerol (DAG), a PKC activator.The activated PKC then phosphorylates target proteins. Thus, like cAMP/PKA, PLC/PKC pathway affects many cellular functions (Walters, 1992).

The stimulatory effects of vitamin D_3 metabolites on phospholipid metabolism in a wide range of cells have been known for some time. For example, $1,25D_3$ increases IP_3 and DAG production and activates PKC in rat colonic epithelium (Wali *et al.*, 1990); induces PKC translocation in the terminal keratinocyte (this has been linked to its effects on the differentiation of these cells) (Yada *et al.*, 1989); and modulates the transcription

of PKC in HL-60 cells (Obeid *et al.*, 1990). $1,25D_3$ has also been reported to affect the IP_3/DAG/PKC activity in kidney cells (Zuzuki *et al.*, 1991), liver cells (Baran *et al.*, 1988) and fibroblasts (Sasaki *et al.*, 1986).

Some actions of vitamin D_3 metabolites on bone have been linked to their ability to active the PKC pathway. In cultured chondrocytes from the growth zone of rat costochondral cartilage, the application of $1,25D_3$ resulted in a rapid, dose-dependent stimulation of PKC activity, while $24,25D_3$ caused a slow activation of PKC activity in resting zone cultures (Sylvia *et al.*, 1993). Both $1,25D_3$ and $24,25D_3$ have been shown to stimulate the production of IP_3 and DAG in confluent mouse osteoblasts as early as 5-10 seconds after application (Grosse *et al.*, 1993). Bone resorption induced by $1,25D_3$ is potently inhibited by staurosporine, a kinase inhibitor, indicating the possible involvement of PKC in the action of vitamin D_3 on bone (Johannes *et al.*, 1992).

The above evidence suggests that changes in IP_3 concentration and PKC activity in response to vitamin D_3 metabolites may be part of the mechanism by which vitamin D_3 metabolites modulate the functions of bone cells. However, some controversial questions remain unanswered: 1) The change of IP_3 concentration and PKC activity are a membrane receptor-mediated phenomenon. Thus, are the effects of vitamin D_3 on IP_3 concentration and PKC activity also membrane receptor-mediated? 2) Do the other nongenomic effects induced by vitamin D_3 in bone, such as the modulation of $[Ca^{2+}]_i$ and voltage dependent calcium channels, relate to and/or interact with IP_3 and PKC activation? The answers to these questions will lead to a better understanding of the nongenomic effects of vitamin D_3.

1.4.2.4. The involvement of G proteins in the effects induced by vitamin D_3 metabolites

The G proteins are a family of proteins present in the cell membrane and serve as membrane-bound transducers coupling membrane receptors to intracellular signalling systems. G proteins have been demonstrated to be intermediates in the activation of PKA and PKC (Freissmuth *et al.*, 1989; Birnbaumer, 1990) and voltage-dependent calcium channels (VDCCs) have been reported to be modulated directly or indirectly by G proteins (Brown *et al.*, 1989; Kanaide *et al.*, 1986; Yatani *et al.*, 1987). G proteins contain three subunits designated α,β,and γ and interact directly with receptors on the surface of the cell (Birnbaumer, 1990). The α-subunit contains a single, high-affinity binding site for guanine nucleotides and possesses the GTPase activity which is crucial for the action of G proteins. Activation of G proteins involves an exchange of guanine nucleotides and the apparent dissociation of the α-subunit from the β and γ-subunits, When a G protein is in the basal state, GDP is bound to the α-subunit. During activation induced by the coupling of a ligand to its receptor, GDP is released and replaced with GTP. Once the α-subunit binds with GTP, it dissociates from the β and γ-subunits and becomes activated. The activated α-subunit can then activate various effectors, such as enzymes. Adenylyl cyclase and PLC are two important enzymes which are activated by G proteins, leading to the activation of PKA, IP_3 and PKC pathways. The α-subunit also contains intrinsic GTPase activity which converts the activated α-GTP to α-GDP, after which α-GDP reassociates with the β,γ-subunits to complete the cycle (Birnbaumer, 1990; Simon *et al.*, 1990; Sternweis and Pang, 1990).

Functionally, G proteins can be divided into several subtypes. G_s is the stimulatory G protein for adenylate cyclase and G_i is the inhibitory G protein for adenylate cyclase, while G_o is the other G protein involved in phosphatidylinositol 4,5-biphosphate (PIP_2) hydrolysis (Simon *et al.*, 1990; Smrcka *et al.*, 1991). GTPγS, a non-hydrolysable GTP analogue and GDPβS, a non-hydrolysable GDP analogue, are often used to investigate the involvement of G proteins in the modulation of cellular events. As stated previously, the calcium channels in many types of the cells have been shown to be regulated by G proteins (Birnbaumer, 1990).

Since PKA, IP_3 and PKC and calcium channels can be quickly modulated by vitamin D_3 metabolites, it is reasonable to assume that G proteins are involved in the rapid actions of vitamin D_3 metabolites. However, little is known about the relationship between vitamin D_3 metabolites and the activation of G proteins in bone cells.

1.4.2.5. The interaction of the intracellular signalling systems

The interaction between intracellular signalling systems is often seen when multiple signalling systems are activated at the same time. It is known that one ligand can either activate a single intracellular second messenger system or multiple second messenger systems. For example, PTH can activate both cAMP/PKA and PLC/PKC systems in bone cells (Klem *et al.*, 1990; Civitelli *et al.*, 1988). Pituitary adenylate cyclase activating polypeptide (PACAP) has also been shown to stimulate dual signalling cascades in PC12 cells (Deutsch *et al.*, 1992). An intracellular signalling system is commonly activated by several agonists. For example, cAMP/PKA in bone cells can be

activated by PTH, calcitonin and PTH-related peptide (PTHrP) (Ferrier *et al.*, 1988, 1992; Kumagai *et al.*, 1989). Therefore, when multiple intracellular signalling systems are activated, integration of these signalling systems occurs. The final biological effect will be determined by the sum of these multiple signalling systems. For example, the voltage-dependent calcium channels in vascular smooth muscle cells are modulated by both cAMP and cGMP signalling pathways (Ishikawa *et al.*, 1993). The integration of multiple intracellular signalling systems may provide a precise control of cellular functions that can accommodate various inputs. Since vitamin D_3 and its metabolites have been shown to activate multiple intracellular second messenger pathways in bone, it is reasonable to speculate that these various signals will be integrated to mediate the actions of vitamin D in bone cells.

In summary, vitamin D_3 metabolites have both genomic and nongenomic effects on many types of cells. The genomic effects of vitamin D_3 metabolites involve the activation of nuclear receptors, gene transcription, and protein synthesis, and require a long time to develop. The genomic effects of vitamin D_3 likely mediate long-term physiological effects on the target cells. In contrast, the nongenomic effects of vitamin D_3 occur quickly and appear to be independent of the activation of nuclear receptors and gene transcription. These effects have been found to modulate several intracellular second messenger systems in bone. The physiological role played by the nongenomic effects still remains unclear. A schematic representation of the genomic and nongenomic effects of vitamin D_3 is shown in Fig.I-3.

1.5. Voltage-dependent calcium channels

Calcium channels provide a major path for extracellular calcium entry into most cells. The activity of calcium channels directly affects the intracellular calcium concentration which is fundamentally important for many cellular functions (Tsien and Tsien, 1990). Calcium channels can be divided into several types according to their electrophysiological and pharmacological properties, including the voltage-dependent calcium channels, receptor-operated calcium channels and second-messenger operated calcium channels. Since this study is focussed on the voltage-dependent calcium channel, the receptor operated and second messenger operated calcium channels are not discussed.

Voltage-dependent calcium channels are the most extensively studied class of calcium channels. Common features of VDCC include steep depolarization-dependent activation (Fox *et al.*, 1987), selectivity by high affinity binding of Ca^{2+} (Tsien *et al.*, 1990). Voltage-dependent calcium channels can be further divided into several subtypes.

1.5.1. L type calcium channel (Long-Lasting calcium channel)

L type calcium channels (L channels) are widely distributed in both excitable and nonexcitable tissues (Tsien and Tsien, 1990). Activated by strong depolarizations, L channels inactivate slowly and are highly selective for Ca^{2+}. Ba^{2+} is more permeable than Ca^{2+} through this type of Ca^{2+} channel. The single channel conductance is approximately 21-28pS. L channels are sensitive to dihydropyridine (DHP) agonists and antagonists but not sensitive to toxins such as ω conotoxin (Campbell *et al.*, 1988; Tsein *et al.*, 1990; Spedding and Paoletti, 1992). The L channels are the major pathway for the entry of calcium involved in the activation of contraction of the heart (Noma *et al.*, 1983) and

most kinds of smooth muscle (van Breemen *et al.*, 1987) and in the control of transmitter release from endocrine cells and sensory neurons (Zucker and Lando, 1986; Rane *et al.*, 1987).

1.5.2. T type calcium channel (Transient or tiny channel)

In contrast to L channels, T channels have different electrophysiological properties than L channels (Tsien and Tsien, 1990). T channels can be activated by small depolarizations and inactivated rapidly. The permeability of Ba^{2+} and Ca^{2+} through this type of channel is similar. The single channel conductance is about 8pS. T channels are not sensitive to DHPs and other channel modulators. T channels are more sensitive to Ni^{2+} and less sensitive to Cd^{2+} than L channels. Since few specific T channel blockers are known, T channel structure remains largely unknown. T channels are found mainly in the heart and neurons. They support pacemaker activity and induce calcium entry into cells at negative membrane potentials (Lee and Tsien, 1984; Fox *et al.*, 1987; Bean, 1989).

1.5.3. N-type channels

N channels, which have been linked to the release of transmitters in neurons, occur only in neurons. Their characteristics fall between those of L and T channels. The activation voltage for N channel is more negative than that for L channels but less negative than that for T channels. N channels inactivate much slower than T channels. N channels are insensitive to DHPs but are sensitive to ω-conotoxin GVIA. The single channel conductance of N channels is about 13 pS which is intermediate between that of T and L channels. N channels have been linked to the release of transmitters in neurons

(Hirning *et al.*, 1988; Tsien and Tsien, 1990; Miller and Fox, 1990).

1.5.4. P type calcium channels

P channels were first identified in Purkinje cells of the cerebellum. The P channels differ from other calcium channels in that they are insensitive to DHPs and ω-conotoxin GVIA but sensitive to two other toxins, FTX and ω-Aga-IVA. The single channel conductance is 10-12 pS. P channels activate at moderately high voltages and show little inactivation. P channels have also been proposed to modulate neurotransmitter release (Linas, *et al.*,1989a; 1989b).

Other types of calcium channels that have been described include receptor-operated Ca^{2+} channels, second messenger operated Ca^{2+} channels, and mechanically operated Ca^{2+} channels (Tsien and Tsien, 1990). These calcium channels have their own properties and distinct functions. Since the focus of this thesis is voltage-dependent calcium channels, these other calcium channels are not discussed in detail in this brief review.

1.5.5. Voltage-dependent calcium channels in bone

VDCC in bone cells have been observed for some time. $^{45}Ca^{2+}$ uptake has been used to show that calcium influx could be elicited by KCl-induced depolarization in cultured bone cells (Yamaguchi, 1987). Fura-2 measurements have also indicated that calcium influx was partially responsible for the increase in $[Ca^{2+}]_i$ stimulated by calcium regulating hormones in bone cells (Ferrier, 1992). Furthermore, this calcium influx could be eliminated by the removal of extracellular calcium or by specific VDCC blockers such

as nifedipine and verapermil, suggesting the presence of VDCC in bone cells (Lieberherr *et al.*, 1981). However, direct electrophysiological evidence was not available until recently. The first report to demonstrate VDCC in bone cells came from Chensroy-Marchais *et al.*(1988). Using the patch clamp technique, they successfully recorded both a slow inward calcium channel current and a transient calcium current in primary bone cells obtained from new-born rat calvaria. Subsequently, UMR-106 and ROS 17/2.8 cells, two rat osteogenic sarcoma cell lines, have also been shown to express L type VDCC (Guggino *et al.*, 1988; Duncan and Misler, 1989; Karpinski *et al.*, 1989; Morain *et al.*, 1992). The VDCCs described in these two cell lines are similar to those described in other types of cells (Tsien *et al.*, 1988; Wang *et al.*, 1989). The L type calcium currents in bone cells are sensitive to Bay K 8644 and nifedipine (Karpinski, 1989; Morain *et al.*, 1992). In these reports, the membrane potentials at which L type calcium channel currents were activated were between -30 and -20mV. The peak inward currents occurred at the membrane potentials between 0 to 40 mV. Single channel conductance was about 20pS (Caffrey *et al.*, 1989), similar to the conductance of L channels from other cells (Tsien *et al.*, 1989). The existence of T type calcium currents in bone cells has been controversial. For instance, the T type channel was found in the primary culture of bone cells and UMR-106 cells (Chensery-Marchais, 1988) but not in ROS 17/2.8 cells (Caffery, 1989).

In addition, it is not clear whether or not the expression of VDCC in bone cells is time- and passage-dependent although this phenomenon has been described in other types of cells (Wang *et al.*, 1989). Furthermore, even though it has been well

demonstrated that the VDCC can be modulated by the second messenger systems, (such as PKA and PKC), in many other cells, this remains unknown in bone cells. Therefore, further characterization of the expression and the regulation of VDCCs by second messengers in bone cells is essential.

The specific objectives of this thesis research are:

1. Characterization of the types of voltage-dependent calcium channels expressed in UMR-106 cells.

2. Investigation of the effects of $24,25D_3$ on voltage-dependent calcium channels in UMR-106 cells.

3. The subcellular mechanisms underlying the effects of $24,25D_3$ on the L type calcium channel in UMR-106 cells.

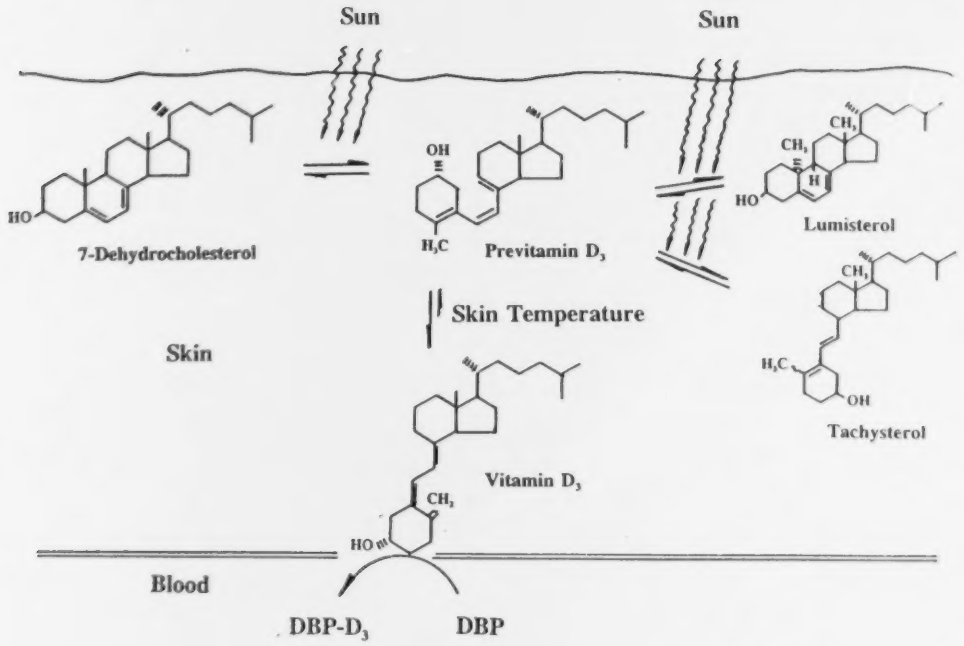

Fig.I-1. Schematic representation of the formation of vitamin D_3 in the skin. During exposure to sunlight, the B ring in 7-dehydrocholesterol is cleaved. This forms previtamin D_3. Once previtamin D_3 is formed in the skin, it immediately begins to equilibrate thermally to vitamin D_3. Once vitamin D_3 is formed, it is translocated into the circulation by vitamin D-binding proteins. During continual exposure to the sun, previtamin D_3 also photoisomerizes to lumisterol and tachysterol which are biologically inert (adapted from Holick, 1989).

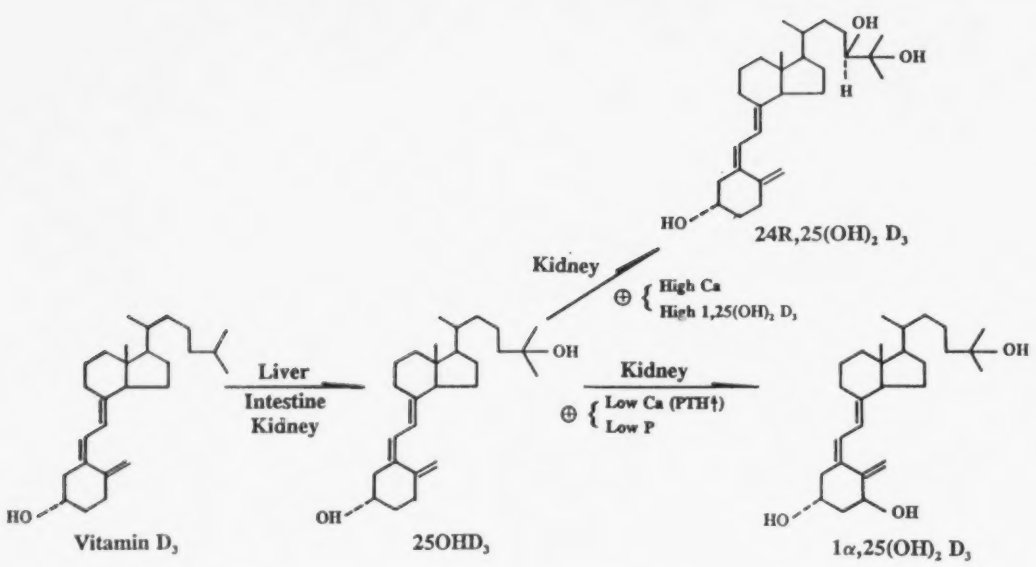

Fig.I-2. Schematic representation of the metabolism of vitamin D_3 to $1\alpha,25(OH)_2$ D_3 and $24,25(OH)_2$ D_3. Vitamin D_3 undergoes its first hydroxylation in liver. The formed 25-(OH)-D_3 is transported to kidney where 25-(OH)-D_3 is converted to $1\alpha,25(OH)_2$ D_3 and/or $24R,25(OH)_2$ D_3. When serum Ca^{2+} and P^+ levels are low and the PTH level is high, the 1α-$25D_3$-dehydroxylase is activated which converts 25(OH) D_3 into $1\alpha,25(OH)_2$ D_3 ; when serum Ca^{2+} and P^+ and $1\alpha,25D_3$ levels are high, 1α-$25D_3$- dehydroxylase is inhibited and 24R-$25D_3$-dehydroxylase is activated which converts 25(OH) D_3 into $24,25(OH)_2$ D_3 (adaptedfrom Holick 1989).

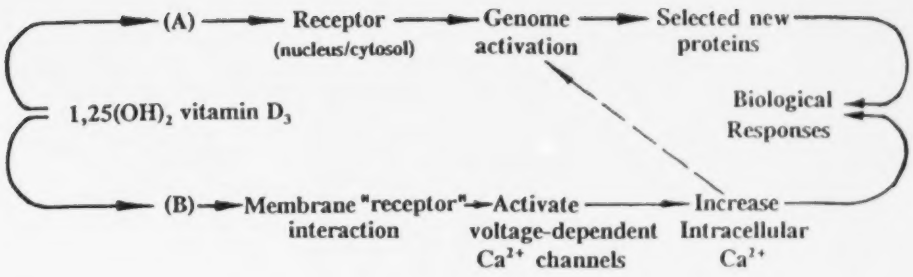

Fig.I-3. Schematic representation of the genomic and the nongenomic actions of vitamin D_3 and its metabolites. The genomic action of vitamin D_3 involves the coupling of vitamin D_3 and its nuclear receptors, gene transcription, and functional protein synthesis. The genomic actions need a long time to develop. The nongenomic actions of vitamin D_3 do not involve gene transcription and protein synthesis and occur very quickly. Vitamin D_3 and its metabolites may bind to a membrane binding site which in turn activates intracellular second messager systems. The activated second messenger systems then modulate many important cellular functions, e.g. phosphorylation of voltage-dependent calcium channels.

Chapter II

Materials and Methods

2.1. Cell preparation

UMR-106 cells were used in this study. The reasons for choosing UMR-106 cells for these studies were as follows: 1) UMR-106 cells are a rat osteogenic sarcoma cell line originating from an osteoblastic sarcoma. Osteoblasts are the main target cells for vitamin D_3 and receptors for vitamin D_3 are widely distributed in the nucleus of these cells (Martin *et al.*, 1976). Additionally, a specific binding site for the vitamin D_3 complex (vitamin D_3 and its cytosolic binding protein) has recently been found in the cell membrane (Kim *et al.*,1994). 2) UMR-106 cells retain all the characteristics of osteoblasts, including alkaline phosphatase and collagen synthesis, as well as cAMP accumulation in response to PTH. Therefore, the UMR-106 cell line is a good model to study the effects of vitamin D_3 and its metabolites on bone.

UMR-106 cells (passages 25-46) were cultured at 37°C in a humidified atmosphere of 5% CO_2 in room air. Dulbecco's Modified Eagle Medium (DMEM) (Gibco) containing 10% fetal bovine calf serum (FCS) (Gibco) was changed every 2 days. The cells usually become confluent after 7 days in culture. After 3 washes with Ca^{2+}-free Hank's buffered saline solution (HBSS) (Gibco), the cells were treated with 0.01% trypsin and 0.25 mM EDTA for 3 to 4 min. The harvested cells were then washed 3 times with DMEM containing 10% FCS, and resuspended in the same medium for use in different experiments. Viability of the harvested cells was greater than 96% tested using trypan blue exclusion. For patch clamp studies, approximately $5x10^4$ cells

were re-plated per 35 mm tissue culture dish in DMEM containing 10% FCS. The re-plated cells were used within 2 to 6 hours. In some experiments designed to study channel expression, the culture times for the re-plated cells were prolonged to 48 or 72 hours before current recordings were performed. For intracellular calcium determinations, approximately $5x10^5$ cells were seeded on glass cover slips with a diameter of 1.5 cm. The cells were cultured in DMEM containing 10% FCS for 24 hours before they were used. For intracellular cAMP determinations, approximately $2.5x10^4$ cells were seeded per well of 24-well culture plates. They were maintained in DMEM with 10% FCS for 48 hours before use.

2.2 Patch clamp technique

2.2.1. Solutions

The compositions of the external (extracellular) solution and the internal (intracellular) solution were designed to separate the voltage-dependent calcium channels currents from other ionic currents. The external solution contained (in mM): Tris, 110; $BaCl_2$,20; CsCl,5; HEPES,20; KCl,5; glucose,20; and TTX,0.5 μM. In all experiments, Ba^{2+} was used as the charge carrier instead of Ca^{2+} for the following reasons. First, Ba^{2+} is more permeable than Ca^{2+} through L-type calcium channels (Fox *et al.*,1987; Tsien *et al.*,1990). Second, Ba^{2+} in the external solution can block Ca^{2+}-activated K^+ channels. Third, Ba^{2+} also suppresses the potassium current through the delayed rectifier channel and the inward rectifier channel (Hagiwara *et al.*,1978; Armstrong *et al.*,1982). Fourth, Ba^{2+} prevents calcium-induced calcium channel inactivation (Armstrong and Taylor, 1980; Eaton and Brodwick, 1980; Akbarali *et al.*,1991). Fifth, the influx of Ba^{2+} during

repeated depolarizations does not inhibit calcium channels (Brown *et al.*,1981: Lee *et al.*,1985;). The use of Tris in the external solution also reduces leakage and prolongs the viability of the cells (Moolenaar and Spector, 1978). The use of Na^+-free bath solution and TTX also eliminated possible contamination by inward Na^+ currents.

The internal solution contained (in mM): Cs_2-aspartate,70; EGTA,10; ATP-Na_2,2; $MgCl_2$,5; K-pyruvate,5; K-succinate,5; glucose,25; HEPES,15; creatine phosphate-Na_2,5; and creatine-kinase,50 units/ml. The advantages of this composition are as follows. First, cesium ions block the potassium channels from the inside of the membrane. Second, K-pyruvate and K-succinate support the citric acid cycle. Third, creatine phosphate-Na_2, creatine-kinase and ATP facilitate the process of energy yield and utilization. Thus, contamination by ionic currents other than calcium current and the "run-down" of the calcium channel current were minimized. Indeed, as a result, in the present study, the calcium current recorded could be maintained from at least 20 min to as long as 2 hours in some cells. Since 10 mM EGTA was included in the Ca^{2+}-free internal solution, the concentration of intracellular free calcium was estimated to be buffered at a concentration of less than 10^{-9} M (Hille, 1974; Hagiwara and Byerly, 1981; Sada *et al.*, 1988). The low concentration of intracellular calcium, on one hand, helped to decrease the calcium-induced inactivation of calcium channel current. On the other hand, it decreased the activity of calcium-dependent enzymes which in turn decrease or delay the effects mediated by these enzymes.

All solutions were filtered through a filter with a pore size of 0.22 μm in diameter before use. The osmolality of all solutions was adjusted to 320-340 mOsm and the pH

was titrated to 7.4 using HCl, CsOH or $Ba(OH)_2$ as required.

The above manipulations ensured that the inward current recorded in the present studies was due to the inward movement of Ba^{2+} through calcium channels.

2.2.2. Whole-cell patch clamp recording

After the UMR-106 cells were washed 3 times with the external solution, the Petri dish with these cells was mounted onto the stage of an inverted phase contrast microscope (Nikon, Diaphot). The patch pipettes were fabricated from borosilicate (omega dot) thin-walled glass tubes (1.2 mm OD, 0.9 mm ID). They were pulled using a two stage microelectrode puller (Narishige PP-83, Tokyo, Japan) and the tip was fire polished using a microforge (Narishige MF-83). The resistance of the pipettes filled with the internal solution used for calcium channel current recording was 2 to 5 megohms. The pipette solution was connected via an Ag/AgCl electrode to an Axopatch-1B (Axon Instruments, Inc., CA) patch clamp amplifier with a 500 megohms feedback resistor in the headstage. The standard gigaohm seal, whole-cell version of the patch clamp technique was used to measure whole-cell inward current (Hamill *et al.*, 1981). Before the cell membrane was touched, the junction potential between the pipette and the bath solution was zeroed by adjusting the pipette current to zero. The pipette was pushed onto the cell surface using a three dimensional micromanipulator (Narishige). A tight seal (gigaseal) between the membrane and the tip of the pipette (10 to 50 gigaohm) was obtained by suction through the pipette. Further suction disrupted the membrane under the tip of the pipette and the pipette solution dialysed the cell. Free access to the cytosol

was reached when a capacitive current appeared and a membrane current could be recorded as a function of test potential.

The experiments were carried out at room temperature (20 to 22°C) in order to ensure a longer survival time of voltage-clamped cells and a better time resolution of the fast membrane currents. In all the experiments, pulses of 200 msec duration were applied at intervals of 10 sec to allow complete recovery of calcium channel inactivation. All signals were filtered at 1 KHz (4-pole low-pass Bessel filter), analog to digital converted and stored on the floppy disk of a personal computer (Zenith Data Systems) using pClamp software (version 5.6) and a DigiData 1200 interface (Axon Instruments).

In order to obtain a good space clamp, cells with a diameter of approximately 30 μm or less were chosen. (The diameters of UMR-106 cells were usually between 10 to 40 μm). The following observations indicated an acceptable temporal and spatial clamp. 1) The capacitive transient (current) settled fast enough to separate it from the relatively slow calcium current. 2) The capacitive current recorded under patch clamp conditions had an exponential decline. 3) The activation, inactivation (T type), and slow inactivation (L type) segments of inward current at a fixed test pulse did not show any abnormal notches (indicative of a poor spatial clamp). 4) The current-voltage (I-V) relationships of T or L channel currents were smooth and bell-shaped without abrupt changes of inward current (Colatsky *et al.*, 1979).

An universal phenomenon in patch-clamp studies is the decline of calcium channel current with time ("run-down"). In UMR-106 cells, immediately after the cell was voltage-clamped, the inward current recorded was usually small. However, the current

increased gradually in the following 3 to 5 min and became relatively stable, as the result of the outward K^+ current being blocked by the high concentration of Cs^+ in the pipette and Ba^{2+} in the bath (Armstrong and Taylor, 1980; Quandt and Narahashi, 1984). If the current magnitude did not change from the third to the fifth minute after the rupture of the cell membrane, the rate of "run down" of the inward current in UMR-106 cells was usually negligible for 30 min or longer. Therefore, only cells which had stable inward currents from the third to fifth minute after penetration of the membrane were used. If the current decayed too quickly, it was difficult to distinguish "run down" from the inhibitory or excitatory effect of the agents. Cells which had a fast decline of inward current within this period were discarded.

Leakage and capacitive currents were subtracted on line with the P/2 protocol using pClamp software. The subpulse used to subtract the leakage current did not activate any ionic current within the test pulse range examined. In the presence of 2 mM La^{2+}, the I-V relationship of the leakage current is linear at negative membrane potentials and shows a minor upward deflection at positive membrane potentials, for which the intracellular Cs^+ may be partially responsible. The product of series resistance and membrane current affect the accuracy of the voltage clamp. When there is a large series resistance, there is a deviation of the membrane potential from the command potential. In the present study, the peak currents were usually small (approximately 20 to 120 pA) with 20 mM Ba^{2+} as the charge carrier, and the series resistance was usually less than 10 megohms. The voltage error due to series resistance was less than 2 mV and, hence, series resistance compensation was not usually employed. In cases where the voltage

error was greater than 2 mV, series resistance was compensated electronically using the Axopatch 1-B electronics.

The control I-V relationships were constructed using the peak values of the inward current (leakage corrected) and the currents were activated by stepwise depolarization of the cell to various test potentials at a frequency of 0.5 Hz. Different agents were then added directly into the bath solution and the change in the inward current was monitored continuously using test depolarizations at a frequency of 0.033 Hz. Since the agents required a period of 1 to 3 min to be distributed evenly throughout the bath solution, the I-V relationships representing the effects of the agents on calcium current were usually obtained after at least a 3 min exposure of the cell to the test agents. A 5% change (higher or lower) in the amplitude of the inward current was considered to be a significant change induced by the agents used in the experiments.

Solution exchange (washout) was performed in some cells in order to determine whether the effects of these agents were reversible. The replacement of the bath solution was carried out using a perfusion system which was able to change the bath solution in 1 min. The washout procedure also helped distinguish the inhibitory effect of agents from "run down" of the calcium channel current.

2.3. $[Ca^{2+}]_i$ determination

An intracellular calcium indicator, Fura-2 AM, was used to measure $[Ca^{2+}]_i$ in UMR-106 cells. The UMR-106 cells were prepared and cultured as described in the section entitled "Cell preparation". After being replated and grown on glass cover slips

for 48 hours, UMR-106 cells became confluent and formed a monolayer. Before loading with Fura-2 AM, the old culture medium (DMEM) was replaced by fresh media and Fura-2 AM dissolved in DMSO was directly added to the medium to achieve a final concentration of 2 μM. The UMR-106 cells in the Fura-2-containing medium were then incubated in the dark at room temperature for 50 min. After removal of the loading medium, the cells were then washed 3 times with a 5K buffer solution which contained (in mM) NaCl 145; KCl,5; MgCl,1; D-glucose,10; $CaCl_2$,2; NaH_2PO_4,0.5; HEPES,10. The pH and osmolality of the 5K solution were adjusted to 7.4 and 320 mOsm, respectively. In order to determine the source of the increased $[Ca^{2+}]_i$, Ca^{2+} was omitted from the 5K solution in some experiments. The cover slips with attached UMR-106 cells loaded with Fura-2 were placed in a 32x7-mm Sykes-Moore chamber and covered with 1 ml 5K solution. The top of the chamber was left open to allow access for the addition of various agents or for buffer exchange. The chamber was then mounted onto a stage of the inverted epifluorescence microscope (Diaphot-TMD). The cells were observed using a Nikon phase-contrast 40x objective. The excitation light was provided by a dual-excitation spectroflurometric system (Spex Industries, Edison, NJ) interfaced to the microscope. The two excitation monochromators were set at 340 and 380 nm. The excitation UV (340, 380) was directed toward the cells by a diachroicmirror with a 400-nm cut-off wavelength. The fluoresence emitted by Fura-2-loaded cells was filtered by a 510 nm interference filter and directed to a photomultiplier. The photomultiplier signal was relayed to an IBM computer, coupled to the spectrofluorometer using CM3000DM program (Spex Industries). The program generates a simultaneous real-time plot of

fluorescence intensity (510 nm) for both excitation wavelengths. The system was programmed to alternate between 340 and 380 nm excitation every second and the integration time was set at 0.3 sec. After subtraction of background noise, the 340/380 ratio was subsequently calculated for each pair of data points. The fluorescence signal was calibrated according to Grynkiewicz *et al.* (1985) using the relationship:

$$[Ca^{2+}]_i \text{ (nM)} = K_d \times (R-R_{min})/(R_{max}-R) \times b$$

where R was the measured ratio, R_{min} and R_{max} were the values of R at very low and saturating concentrations of Ca^{2+} respectively, and b was the ratio of emission intensities at 380 nm excitation under conditions of very low and saturating concentrations of Ca^{2+}. The K_d was the dissociation constant for Fura-2 and Ca^{2+} and was assumed to be 224 nM (Grynkiewicz *et al.*, 1985). R_{max} was obtained by adding 2 μM ionomycin to the bath solution and R_{min} by the further addition of 10 mM EGTA.

2.4. cAMP measurement

The cells were prepared as described above. Before the experiments, the culture medium was changed to DMEM with 0.1% BSA and the cells were equilibrated in this medium for 60 min. The cells were then incubated with different agents in the same medium for 15 min. To determine cellular cAMP, the drug-containing DMEM was replaced by ice cold 5 mM acetic acid after 15min. In order to release the intracellular cAMP, the cells was lysed by repeated freezing and thawing twice in 5 mM acetic acid. The lysates were then transferred to centrifuge tubes and boiled for 5 min. The samples were stored at -20°C until analysis. The cAMP content was determined with a

radioimmunoassay kit, using ^{125}I-cAMP as the label.

2.5. Drugs

$24,24D_3$ and $1,25D_3$ were a kind gift from Kureha Chemical Industry Co., (Tokyo, Japan). They were dissolved in 95% ethanol and stored in a container protected from light. Bovine PTH (1-34) was purchased from Bachem Inc. (Torrance, CA) and dissolved in distilled water. Bay K 8644 (Calbiochem) and nifedipine (Sigma) were dissolved in DMSO. Dibutyryl adenosine 3'5'-cyclic monophosphate (db-cAMP) and 8-bromo adenosine cyclic monophosphate (8-bromo-cAMP) were purchased from Sigma and were dissolved in DMSO. Rp-cAMPs and GDPβS were purchased from Biolog Life Science Institute (FRG) and dissolved in distilled water. PMA, OAG and Staurosporine were obtained from Sigma and dissolved in ethanol. Calphostin C was obtained from Calbiochem (La Jolla, CA) and dissolved in DMSO. The required concentration of the agent in the bath solution was achieved by adding the concentrated (1000x) stock solution to the dish. The final concentrations of the solvents (ethanol and DMSO) did not exceed 0.1%. This concentration of solvents had no detectable effect on the signals of interest in patch clamp and Fura-2 experiments. The other agents used in this thesis research were of the highest grades available from Sigma.

2.6. Statistics

Results are expressed as the means $\pm$ the standard error of the mean (SEM). The peak inward current obtained from the current voltage relationship was used to determine

the effect of the test agents. The paired Student's t-test or grouped t-test was used for comparison between mean values of the control and those obtained after drug administration. Analysis of variance in conjunction with the Neuman-Keul's test was used for multiple group comparisons. P values less than 0.05 were considered statistically significant.

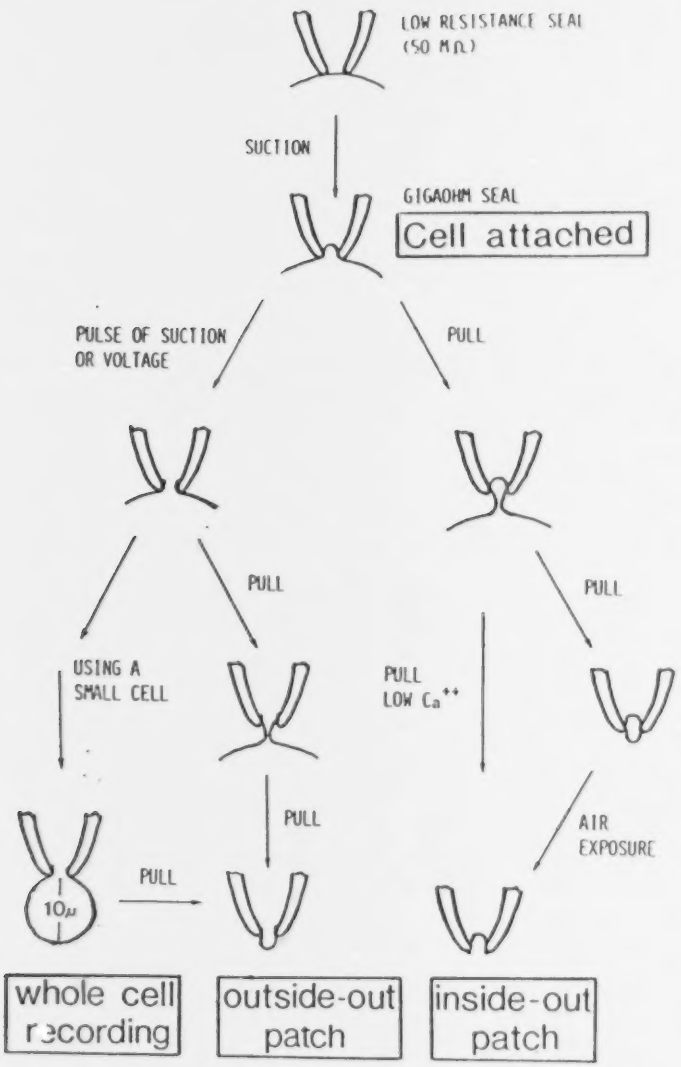

Fig.II-1. Schematic representation of the patch clamp technique. The patch clamp technique consists of two versions: the whole cell and the single channel version. The single channel version can be further divided into cell attached, outside-out patch and inside-out patches (adapted from Hamill *et al.*, 1981).

Chapter III

Expression and characteristics of voltage-dependent calcium channels in UMR-106 cells

3.1 Introduction

It has been speculated by many investigators that voltage-dependent calcium channels are expressed in bone cells (Civitelli *et al.*, 1989). Based on $^{45}Ca^{2+}$-uptake experiments, it has been reported that calcium uptake by osteoblasts (human and rat) is increased by KCl stimulation. KCl depolarizes the cell which, in turn, activates the voltage-dependent calcium channels (Yamaguchi *et al.*, 1989). In later experiments, it was demonstrated that the increase of $^{45}Ca^{2+}$ uptake induced by KCl in bone cells could be blocked by eliminating the extracellular calcium or by the use of a VDCC antagonist (nifedipine). These observations suggest that the voltage-dependent calcium channels are expressed in bone cells.

Experiments using the Fura-2 technique support this hypothesis. Kumagai *et al.* (1992) demonstrated that the intracellular calcium concentration in UMR-106 cells could be increased by the application of peptide hormones and neurotransmitters (*i.e.* PTH and ATP). The increases in intracellular calcium were totally or partly dependent on extracellular Ca^{2+}, suggesting that calcium influx is one source of the increase in intracellular calcium concentration.

Using the patch clamp technique, two types of voltage-dependent calcium channels have been identified in primary cultured bone cells and bone cell lines. Karpinski *et al.*, (1989) reported that an inward calcium current with a slow inactivation rate could be activated from a holding potential of -40mV in UMR-106 and ROS 17/2.8 cells. This slow inward current was sensitive to DHPs (Karpinski *et al.*, 1989). Caffrey and Farach-Carson (1989) reported a similar result using ROS 17/2.8 cells. Another type of VDCC was also recorded in UMR-106 cells (Morain *et al.*, 1993). This current was characterized by fast activation and fast inactivation. A more negative holding potential was necessary for the activation of this calcium current and this current was not DHP sensitive (Morain *et al.*, 1993).

Despite the advances in the understanding of voltage-dependent calcium channels in bone cells, many important aspects of calcium channels in bone remain unclear and controversial. For example, the channel density of T and L currents in bone cells has not been studied. Reports on the differential expression of T and L channels in bone cells are conflicting. Since the main objective of this thesis research was to investigate calcium channels and their modulation by vitamin D_3 metabolites, a detailed characterization of the voltage-dependent calcium channels in UMR-106 cells was necessary. The following experiments were carried out to define the expression and the electrophysiological characteristics of the voltage-dependent calcium channels in this cell line.

3.2.Experimental Design

The whole cell version of the patch clamp technique was used in these experiments. Two holding potentials (-40mV and -80mV) were used to activate and separate the voltage-dependent calcium channels. Ba^{2+} was used as the charge carrier. TTX and Cs^{+} were included in the bath and the pipette solution to eliminate sodium and potassium currents. Bay K 8644 and nifedipine were used to test the sensitivity of the inward calcium channel currents to DHPs.

To investigate the expression of the voltage-dependent calcium channels as a function of culture time and the number of subculture passages, patch clamp experiments were performed during different passages and after the culture time had been prolonged at the higher passages (42-46th).

3.3. Results

3.3.1. Inward calcium currents

Inward calcium currents were elicited from two holding potentials (-80mV and -40mV). The inward Na^{+} channels were blocked by TTX in the bath solution and the exclusion of Na^{+} from the bath solution. The K^{+} currents were blocked by Cs^{+} in the pipette solution and the high concentration of Ba^{2+} in the bath solution. Hence, the recorded inward current was due to Ba^{2+} moving through calcium channels.

3.3.1.1. The L type calcium channel current

When the holding potential was set at -40 mV, one type of calcium current could be activated by the step wise depolarizing of the membrane. Fig.III-1 shows a series of records of this inward calcium current from a UMR-106 cell. This type of inward calcium current had a very slow inactivation time-course. As the test potentials became more positive, the inactivation rate increased. In a group of 12 cells, the half-time of inactivation was > 200 msec at a test potential of 10 mV, and 175 ± 15 msec at a test potential of 30 mV. The half-time of activation at a test potential of 10 mV was 15 ± 2.5 msec in the same group of cells. This current was activated at a membrane potential between -30 to -20 mV and the peak inward current occurred at 10 to 30 mV.

This current was sensitive to DHPs. Fig. III-2 shows that at a concentration of 1×10^{-6}M, Bay K 8644 increased the peak current amplitude by $220 \pm 22\%$ ($n=6$, $p<0.05$); at a concentration of 1×10^{-6}M, nifedipine decreased the peak amplitude of the current by $85 \pm 2.5\%$ ($n=6$, $p<0.05$). Fig. III-3 shows the current records obtained before and after the application of Bay K 8644 and nifedipine in one UMR-106 cell. In this cell, Bay K 8644 increased the peak current amplitude from 109pA to 210pA. Nifedipine decreased the peak current amplitude from 210pA to 29pA. The peak of the I-V relationship was shifted towards to the more negative potentials. In a group of 8 cells, the peak of the I-V relationship was shifted towards negative potentials by about 15mV (data not shown).

3.3.1.2. The T type calcium channel current

When the membrane potential was set at -80 mV, another inward calcium current could be activated from some cells at the higher passages. This inward calcium current was characterized by fast activation and fast inactivation. Fig.III-4 shows a series of records of this current from a UMR-106 cell. The half-activation time obtained from 6 cells was 11 ± 2 msec and decayed with a half-inactivation time of 40 ± 8 msec at a test potential of -10 mV. The peak inward current occurred at -20 to -10 mV. This current was not sensitive to DHPs. Fig.III-5 shows the current traces recorded before and after the application of Bay K 8644 and nifedipine in one cell. In this cell. Bay K 8644 at a concentration of $1x10^{-6}$M did not change the current. At a very high concentration ($1x10^{-5}$M), nifedipine decreased the current amplitude slightly (15%). The peak of the I-V relationship was not changed by either Bay K 8644 or nifedipine. Steady state activation and inactivation experiments were conducted to determine the dependence of activation and inactivation on membrane potential using tail currents. The pulse protocols are shown in the insets of Fig.III-6. The potential at which one-half of the channels were inactivated was estimated to be -50mV and the potential at which one-half of the channels were activated was estimated to be -35mV. Steady state inactivation and activation were not changed by Bay K 8644 or nifedipine. The electrophysiological and pharmacological characteristics of the L and T type calcium channel are summarized in Table III-1.

3.3.2. The passage-dependent expression of the T and L type calcium channels

To examine whether the expression of T and L channels in UMR-106 cells is

related to the cell passage, the recording of T and L currents at different passages were compared. It was found that the expression of the T and the L type calcium channels was passage-dependent. Two different types of expression of calcium channels were observed in this study: a predominant L channel expression and a co-expression of T and L channels. Predominant L channel expression was found in cells from early passages. In a group of 27 cells from passages 24 to 28, only 2 cells (7%) had T currents while the L currents were recorded from 22 cells (81%). The co-expression of T and L channels was found in cells from higher passages. In a group of 32 cells from passages 42 to 46, the T currents were recorded in 10 cells (31%) and the L currents were recorded in 28 cells (87%). The expression of T channel between low and high passages was significantly different ($p<0.05$) while there was little change in the expression of L channel, suggesting that there was an increase in the expression of the T type calcium channels at the high passages while the expression of L type calcium channel was not influenced by the cell passages. Table III-2 summarizes the passage-dependence of the expression of T and L channels in UMR-106 cells.

3.3.3. The culture time-dependence of the channel density

Current densities which are an estimate of channel density were measured using cell capacitance as a measure of cell surface area. The dependency of current densities in UMR-106 cells from higher passages on the culture time was investigated. The result is shown in Fig. III-8. In these cells, during the first 10 hours after replating, the density of the T and the L channel currents were 6.7 ± 1.1 pA/pf and 1.2 ± 0.5 pA/pf,

respectively. The T channel current had a higher density than that of the L channel current at this time. As the culture time was prolonged, the density of the T channel current decreased while the density of the L channel current increased gradually over the entire period of culture. In 48 to 72 hours after replating, the density of the T channel current was lower than that of the L channel current. At this time, the densities of the T and the L channel currents were 1.4 ± 0.4 pA/pf and 1.9 ± 0.4 pA/pf, respectively. The original current records obtained from three different cells at different time are shown at the top of Fig. III-8.

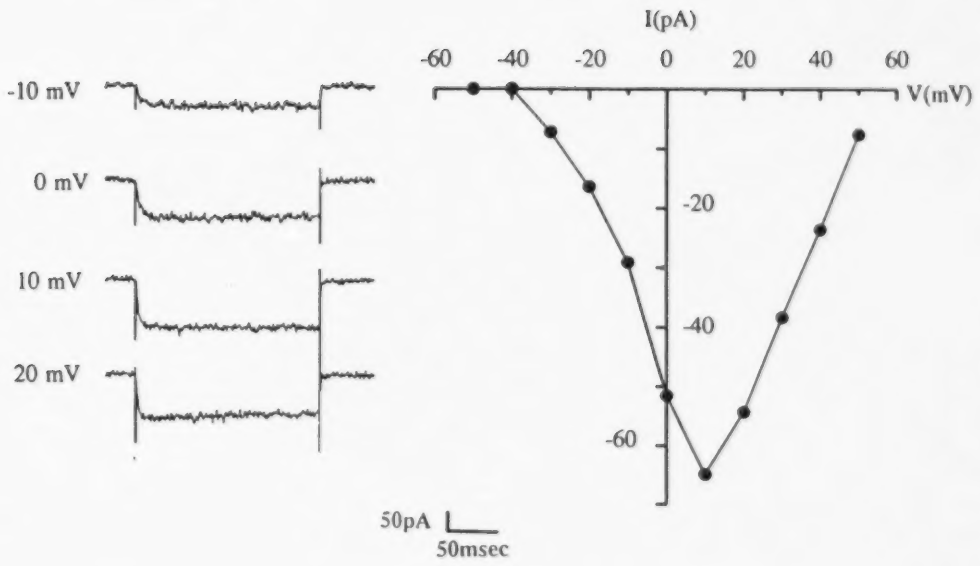

Fig. III-1. The L type Calcium channel current in a UMR-106 cell. The calcium channel currents were activated by depolarizing the cell from a holding potential of -40mV to more positive test potentials. The current records shown on the left were evoked by 200 msec test pulses to the potential indicated next to each record. The leakage currents were subtracted on line. The current-voltage (I-V) relationship for this cell is shown on the right. The current was activated at -30mV and the peak inward current occurred at +10mV.

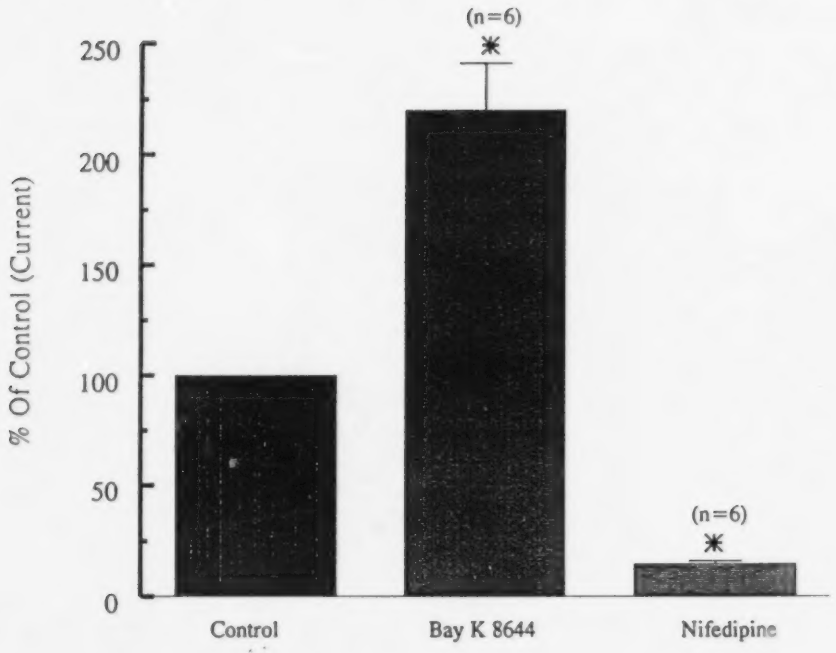

Fig. III-2. Effects of Bay K 8644 and nifedipine on the L type calcium channel in a group of UMR-106 cells. At a concentration of $1x10^{-6}M$, Bay K 8644 increased the peak amplitude of the L type calcium current to $220\pm22\%$ (n=6); nifedipine $1x10^{-5}M$ decreased the peak amplitude of the L type calcium current to $15\pm2.5\%$ of the control level (n=6) (* $p<0.05$).

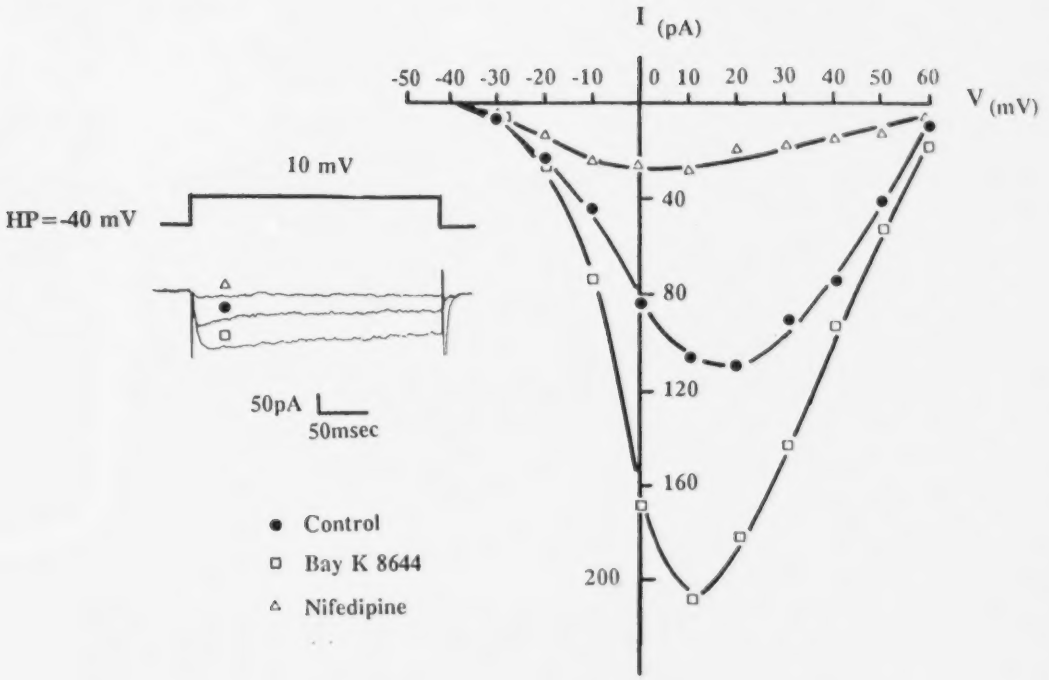

Fig. III-3. Effects of Bay K 8644 and nifedipine on the L type calcium channel current in a UMR-106 cell. The current records shown at the left were activated by depolarizing the cell to +20mV from a holding potential of -40mV. The control current (filled circles), the current 5 min after the application of $1x10^{-6}$M Bay K 8644 (open squares) and the current 5 min after the application of $1x10^{-6}$M nifedipine (open triangles) are shown. The I-V relationships before and after the addition of Bay K 8644 and nifedipine are shown at the right. Bay K 8644 increased the peak current amplitude from 109pA to 210 pA and shifted the peak of the I-V relationship curve from +20 mV to +10 mV. Nifedipine decreased the peak current amplitude from 210 pA to 28 pA. The effect of Bay K 8644 was more pronounced at negative membrane potentials.

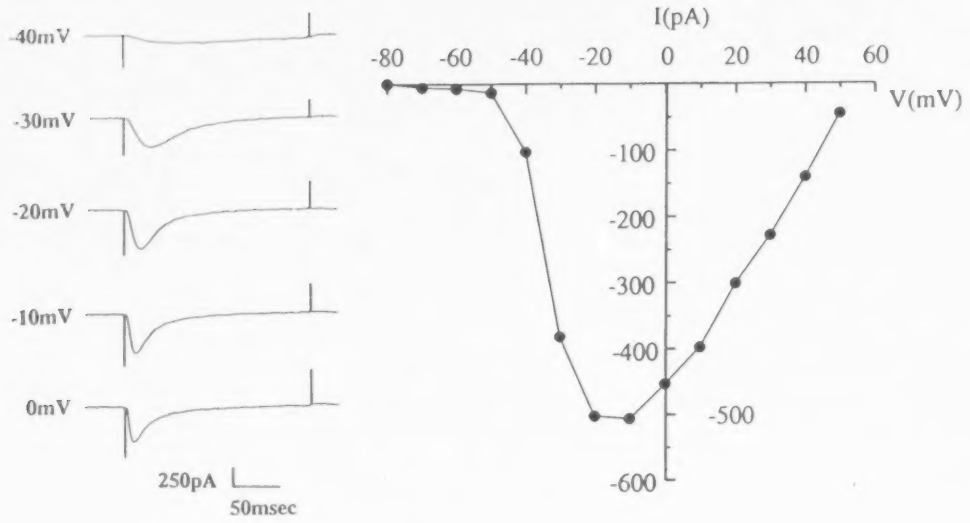

Fig. III-4. The T type calcium channel current in a UMR-106 cell. The T channel current was activated by depolarizing (200 ms duration) the cell from a holding potential of -80 mV to more positive test potentials. The magnitudes of the test depolarizations are indicated next to the currents. The currents were leakage correction on line. The T currents were activated at a potential between -50 and -40 mV and the peak inward current occurred at -10 mV. The current was characterized by fast activation and fast inactivation. The current-voltage (I-V) relationship corresponding to the currents is shown on the right.

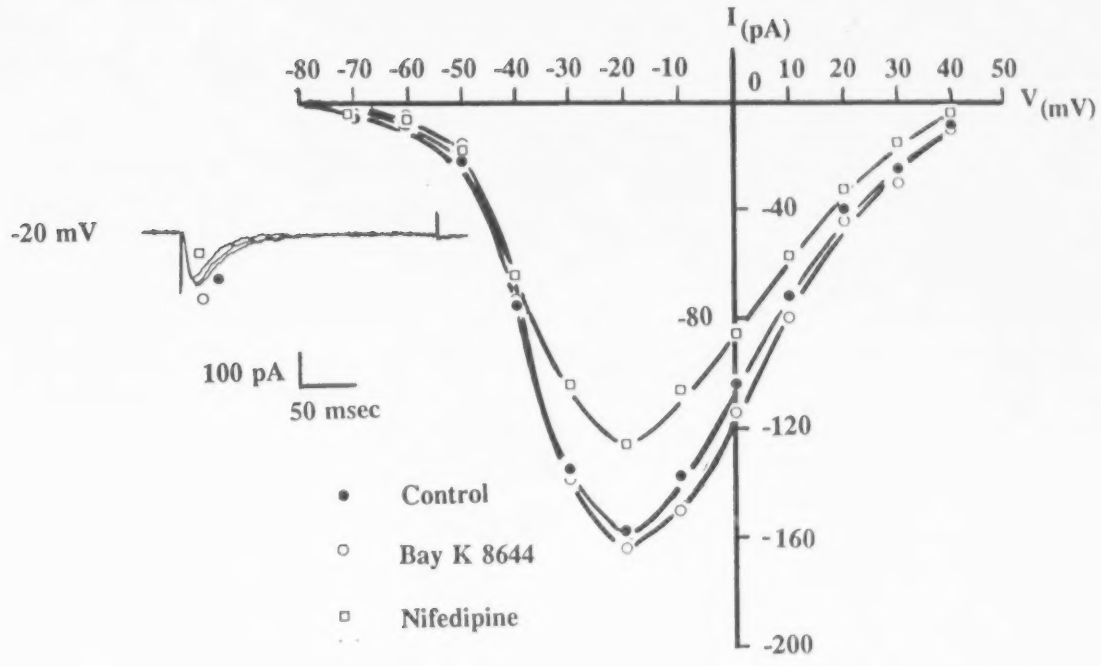

Fig. III-5. The effects of Bay-K 8644 and nifedipine on the T type calcium channel current in a UMR-106 cell. The current records shown at the left were activated by depolarizing the cell from a holding potential of -80 mV to -20 mV . The currents shown are the control current (filled circles), the current 5 min after the application of $1x1^{-6}$M Bay-K 8644 (open circles) and the current 5 min after the application of $1x10^{-5}$M nifedipine (open squares). The I-V relationships before and after the addition of Bay-K 8644 and nifedipine are shown on the right. Bay K 8644 did not affect the current. However, at a high concentration of $1x10^{-5}$M, nifedipine decreased the peak inward current by 15% but did not shift the peak of the I-V relationship.

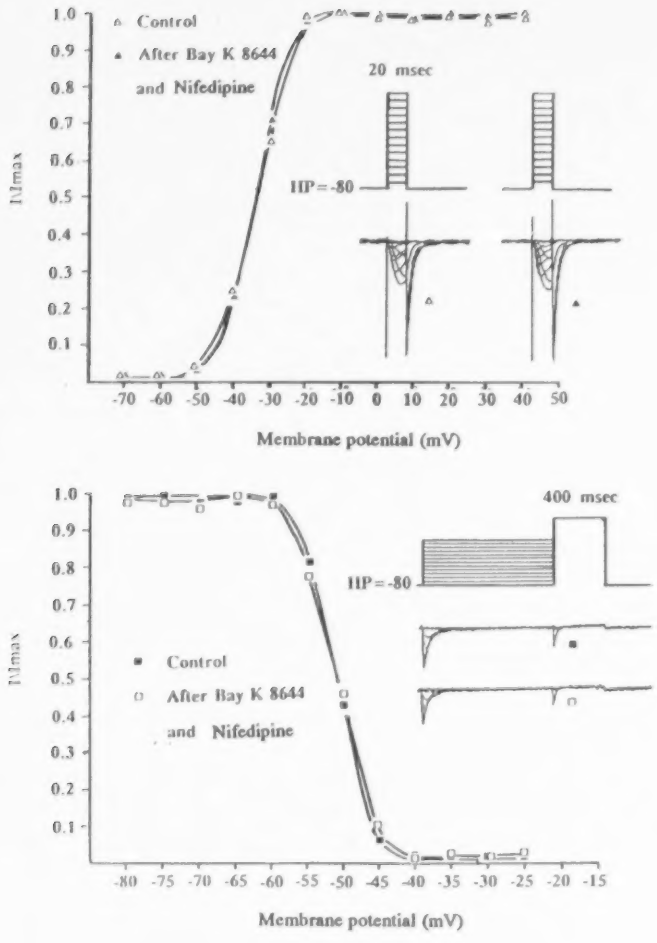

Fig. III-6. Steady-state activation (triangle) and steady-state inactivation (square) of the T type calcium channel currents before (filled triangles and squares) and after (open triangles and squares) Bay K 8644 and nifedipine in a UMR-106 cell. The magnitudes of Imax and I were determined from the tail currents as shown in the insets. The top panel shows the steady-state activation curves and the bottom panel shows the steady-state inactivation curves. The curves were fitted by eye. The steady-state activation and inactivation of the T type calcium channel currents were not changed by Bay K 8644 or nifedipine.

Table III-1: The electrophysiological and pharmocological characteristics of the L and T type calcium channel currents in UMR-106 cells

L type calcium channel	T type calcium channel
Fast activation	Fast activation
Slow inactivation	Fast inactivation
Low threshold	High threshold
Small depolarization for activation	Strong depolarization for activation
Peak current occurs at positive membrane potential	Peak current occurs at negative membrane potential
Dihydropyridine sensitive	Dihydropyridine insensitive

Table III-2: The passage-dependent expression of L and T type calcium channels in UMR-106 cells

Predominant expression of L type calcium channel	Co-expression of L and T type calcium channel
In cells from early passages (passage 24 to passage 28)	In cells from higher passages (passage 42 to passage 46)
Cells expressing L type calcium channel 81% (22 of 27 cells tested)	Cells expressing L type calcium channel 87% (28 of 32 cells tested)
Cells expressing T type calcium channel 7% (2 of 27 cells tested)	Cells expressing T type calcium channel 31% (10 of 32 cells tested) ($p < 0.05$ compared with the cells expressing T type calcium channel in early passages)
L type calcium channel density remains stable during subculture time	T type calcium channel density decreased during subculture time

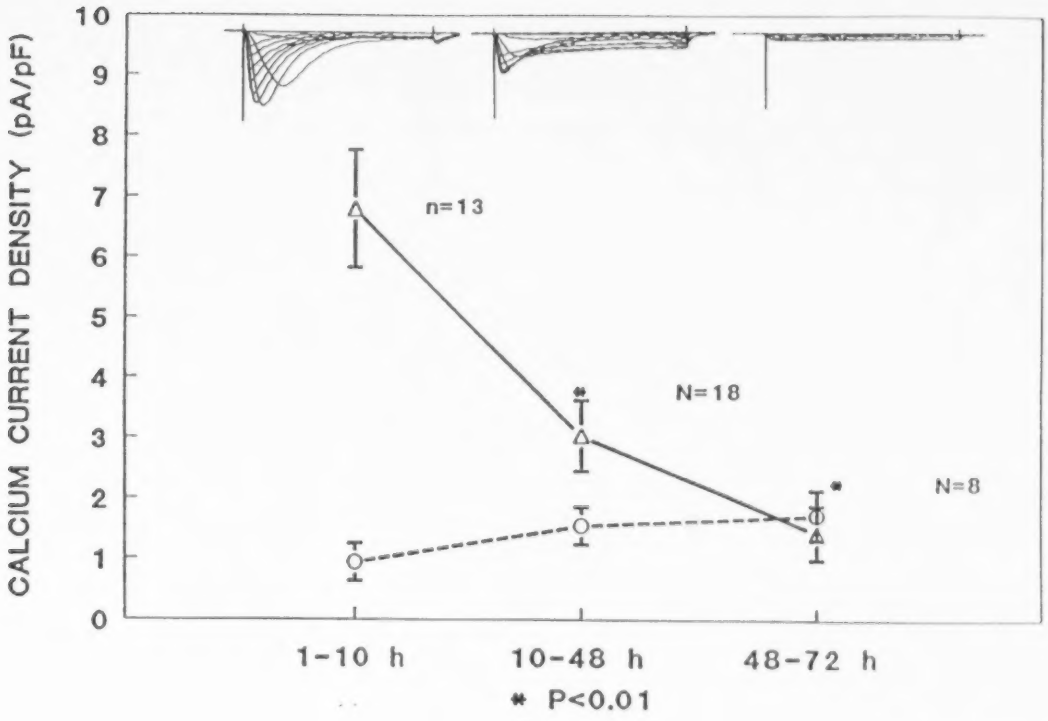

Fig. III-7. The culture-time dependence of the expression of the T and the L type calcium channel currents in UMR-106 cells. The data shown were obtained from cells at passages 42 to 46. The expression of the T type calcium channel current (open triangle) and the expression of the L type calcium channel current (open circle) are shown as current densites. In the first 10 hours, the cells expressed a high density of the T type calcium current and a low density of the L type calcium current. In a group of 13 cells, the current density of the T type calcium channel was 6.7 ± 1.1 pA/pF and 1.2 ± 0.5 pA/pF for the L type calcium channel. As the culture time after the cells were replated was prolonged, the T type calcium channel current density decreased and the L type calcium channel current density increased. 48-72 hours after the cells were replated, in a group of 8 cells, the T type calcium channel density was 1.4 ± 0.4 pA/pF and the L type calcium channel density was 1.9 ± 0.4 pA/pF, respectively. Three currents representing the expression of the T and L type calcium channels are shown at the top of the figure. These currents were obtained from 3 individual cells at different culture times corresponding to the time points of the current density curves.

3.4. Discussion

3.4.1. The characteristics of the T and L type calcium channels in UMR-106 cells

3.4.1.1. The expression of the T and L type calcium channels in UMR-106 cells.

The expression of voltage-dependent calcium channels in bone cells has been documented in mouse MC3T3-E1 osteogenic cells (Amagai and Kasai, 1989); ROS 17/2.5 cells (Guggino *et al.*,1988); UMR-106 cells (Karpinski *et al.*, 1989; Duncan and Misler, 1989; Morain *et al.*, 1992). Both L and T type calcium channels are expressed in bone cells. The characteristics of the L and T channels in bone cells are similar to those found in other cell types (Tsien and Tsien, 1990). For example, the L channel currents recorded from primary cultured bone osteoblast (Chesnoy-Marchais and Fritsch, 1988), ROS 17/2.8 cells (Caffrey and Carach-Carson,1989) and UMR-106 cells (Karpinski *et al.*, 1989) are DHP-sensitive and have a slow inactivation rate. The T type calcium channel currents recorded from ROS 17/2.8 cells (Ferrier,1989) and UMR-106 cells (Morain *et al.*, 1992) are not sensitive to dihydropyridine. The L and T type calcium currents recorded from our preparation have similar characteristics. However, there has not been a consensus of some of the properties of T and L channels in bone cells as summarized here.

3.4.1.2. The current-voltage relationship

The current-voltage (I-V) relationship is an important property used to characterize ion channels (Ferrier, 1989). An I-V relationship is obtained by applying

brief test pulses of different amplitudes from a holding potential. The measured I-V relationship reflects the effect of membrane potential on the activation of a specific ion channel, as well as on the force driving ions through the channel. This depends on both the membrane potential and the difference in ion concentration across the membrane. Therefore, the I-V relationship for a given type of channel depends on the characteristics of the channel itself, and on the concentration of the permeant ion. Comparing the I-V curves reported by different investigators, some differences are noted under similar experimental conditions. Chesnoy-Marchais and Fritsch (1988) reported that two types of calcium channel currents could be recorded in new born rat calvaria cells: a transient and a sustained calcium current with different electrophysiological properties. From a holding potential of -67 mV, a sustained calcium current could be elicited. The peak of this sustained current occurred at about +40 mV. A transient calcium current could also be recorded using the same holding potential. The peak of the I-V curve of this transient current reached a maximum at about +10 mV. The peaks of both the T and L channel currents occurred at positive membrane potentials. In contrast to Chesnoy-Marchais and Fritsches's results, the membrane potential for maximum activation of the T and L calcium currents reported by other groups were more negative. Morain *et al.* (1992) showed that the peak current of the T channel occurred at a membrane potential of -20 mV with a holding potential of -80 mV and the peak current of the L channel occurred at a potential of +20 mV with a holding potential of -40 mV. A similar result was reported by Karpinski *et al.* (1989) and Grygorczyk (1989) reported that, in ROS17/2.8 cells, the peak of the L channel current occurred at a potential of +10mV with a holding

potential of -60mV.

In agreement with these observations, our data showed that the peaks of the T current occurred at a membrane potential of -20 to -10 mV (holding potential of -80mV), and the peak of the L channel current at a potential of +10 to +30 mV (holding potential of -40 mV). When the holding potential for T channel was set at the more negative level (*i.e.* -80 mV), the peak current appeared to occur at a more negative membrane potential. At a holding potential of -45 mV, 100% of the T channels were inactivated (Fig. III-6).

3.4.1.3. The amplitude of the L and T channel current

There is a wide variation in peak current amplitudes of the T and L channels in different bone cell preparations reported by different investigators (Grygorczyk *et al.*, 1989; Caffrey and Farach-Carson, 1989; Karpinski *et al.*, 1989; Morain *et al.*, 1992). In this study, the peak amplitudes of the T and L channel currents in UMR-106 cells were 325 ± 120 pA (n=9) and 120 ± 54 pA (n=14), respectively. The difference in these observations may be attributed to the different holding potentials used in the experiments. For example, -67 mV was used to record both the T and L calcium channel currents in Chesnoy-Marchais's experiments (Chesnoy-Marchais *et al.*, 1988); -60 mV was used to elicit the L calcium current in Czeslawa's study (Czeslawa *et al.*, 1989) and -80mV and -40 mV were used to separate the T and L calcium currents by Karpinski *et al.* (1989).

As stated earlier, at a potential of -40 mV, most of the T channel current is inactivated. Thus, when a holding potential is positive to -40 mV, the T channel is

largely inactivated and only the L channel is activated. In contrast, when the holding potential is more negative, both the T and L calcium channel currents are activated. Therefore, the current amplitude is increased due to the summation of the T and L channel currents. Another possible explanation is that different cell lines and different culture conditions were used in these studies. Even though ROS 17/2.8 and UMR-106 cells are derived from osteogenic sarcoma cells and have major similarities, differences in the expression of the receptors for vitamin D_3 and PTH have been described in these two cell lines (Civitelli and Avioli, 1994). Therefore, the channel density may also be different in these cells. A third possibility is that the culture conditions had an effect on the expression of the type and the density of channels. To properly compare currents from different studies, one needs to compare the current densities.

3.4.1.4. The sensitivity of the L and the T type calcium channel to dihydropyridines

As reported previously (Hess *et al.*, 1984; Bean *et al.*, 1986; Caffrey *et al.*, 1986), DHPs modulate voltage-dependent calcium channels in many tissues. DHPs such as Bay K 8644 and nifedipine have been used as an agonist and antagonist, respectively, to characterize voltage-dependent calcium channels. The sensitivity of the T and L calcium channels in UMR-106 cells to these two DHPs were examined. The actions of these two DHPs on calcium channels in UMR-106 cells can be summarized as follows: 1) Bay K 8644 increased the L type calcium current 2 to 3 fold but had no effect on the T type calcium current. 2) Nifedipine potently inhibited the L type calcium current, but

only slightly inhibited the T type calcium current at high concentrations. 3) The peak of the I-V curve of the L type calcium channel current was shifted 10 to 20 mV towards more negative potentials. 4) At test potentials ranging from -10 mV to +20 mV, the increase in the current caused by Bay K 8644 was more potent. 5) The half-activation and half-inactivation potentials of the L channel currents were shifted towards more negative potential by Bay K 8644. 6) Bay K 8644 reduced the inactivation rate at the test pulses ranging from -20 to +20 mV. 7) Both the enhancing effect of Bay K 8644 and the inhibitory effect of nifedipine on the L calcium channel current were time-dependent and returned to basal levels after the maximal effects were maintained for certain time. However, the inhibitory effect induced by nifedipine was longer lasting than the enhancing effect induced by Bay K 8644. Our observations are in agreement with other studies in bone cells. In Duncan's report, Bay K 8644 was found to increase the opening time and the activity of the L type calcium channel, but not the conductance (Duncan and Misler, 1989). These characteristics described above resemble those of L type calcium channel currents found in excitable and nonexcitable cells (Tsien and Tsien, 1990).

3.4.1.5. The run-down of the calcium channel in UMR-106 cells

The run-down of calcium channel currents has been observed in a number of cells, including bone cells. The reasons for run-down may be due to many factors. For example, some factors, such as small molecules (*i.e.* ATP) inside the cell that are required to maintain the calcium channel activity, are diluted due to perfusion by the pipette solution. The rate of run-down of the calcium channels in bone cells varies from

the first minute to several minute after the whole cell recording configuration is established (Ferrier *et al.*, 1988; Maron *et al.*, 1989). Compared with other observations, the run-down was less pronounced in our preparation. In our experiments, the current usually was small during the first few test pulses and increased gradually in the following 2 to 4 min. This was due to the inhibition of the outward K^+ currents by Cs^+. In most cells, this current amplitude remained stable for at least 20 to 40 min, and in some cells up to 2 hours. This reduced run-down rate could be attributed to the following: 1) A very low concentration of trypsin (0.01%) was used to remove the cells from the culture dishes and minimized the damage to the cell membrane during the procedure. 2) Cs^+-aspartate and EGTA were included in the pipette solution and act as strong chelators for Ca^{2+} and Ba^{2+}. This keeps the Ca^{2+} or Ba^{2+} at a low level and helps to decrease the run-down of the current. 3). ATP, K^+-succinate, K^+-pyruvate, and phosphokinase were also used and provided the cell with an energy source and this may help to stabilize the calcium channel currents.

3.4.2. The passage-dependent expression of the T and the L type calcium channel

The expression of voltage-dependent calcium channels in some cell types has been shown to be time-dependent. In smooth muscle cells from the rat tail artery, it was found that the voltage-dependent calcium channels were detected in the first 24 hours after dissociation and were reduced by 72 hours (Wang *et al.*, 1989). In osteoblasts from the new born rat calvaria, it has been observed that the ratio of the sustained current amplitude over the transient current amplitude increased with the time in culture

(Chesnoy-Marchais and Fritsch, 1988). In agreement with these observations, we found that the expression and the channel density of the T and L type of calcium channels in UMR-106 cells were passage- and culture time-dependent. The T channel currents were seen in cells from the higher passages and had the greatest channel density in the first 12 hours after subculture. The current density declined to a low level in the following 72 hours. In contrast, the L type calcium channels were expressed at all passages. The density of the L channel currents showed a small but gradual increase as a function of the time in culture. This may reflect the different roles played by the L and T channels in UMR-106 cells. Since the L type calcium channels were expressed in cells from all passages, this suggests that the L type calcium channel may play a major physiological role in bone cells. In support of this hypothesis, it has been reported that Bay K 8644, a specific L channel agonist, enhanced bone resorption and nifedipine, a potent L channel antagonist, blocked bone resorption in fetal rat bones (Guggino *et al.*, 1989). Furthermore, some bone-modelling hormones and steroids have been shown to affect the L type calcium channels in bone cells. It has been observed that an increase in intracellular Ca^{2+} in bone cells was an early action induced by many calciotropic hormones (de Boland *et al.*, 1986). For example, the $[Ca^{2+}]_i$ increase induced by $24,25D_3$ required extracellular Ca^{2+} and could be blocked by nifedipine (Lieberherr, 1981). PTH increased $[Ca^{2+}]_i$ from two sources, outside and inside: a small component (30%) of Ca^{2+} release was from intracellular stores and a major component required extracellular calcium and could be blocked by nicardipine, verapamil or La^{2+} (Yamaguchi, *et al.*, 1987a). These results indicate that the effect of several calciotropic hormones in bone is

through their modulation on the L type calcium channels.

The physiological and pathological roles played by the T channel in bone cell functions are not clear at this time. It has been noted that some characteristics and functions of bone cells are related to cell cycle and culture conditions. For example, the alkaline phosphatase activity and osteocalcin secretion are significantly decreased in bone cells from high passages (Chavassieux *et al.*, 1990). Since the T type calcium current is mainly expressed in the cells from higher passages, it is possible that T channels may control alkaline phosphatase activity and osteocalcin secretion.

Chapter IV

The effects of 24,25(OH)$_2$ Vitamin D$_3$ on the L type calcium channel in UMR-106 cells

4.1. Introduction

Vitamin D$_3$ and its metabolites are seco-steroid hormones which play an important role in calcium homeostasis due to their actions on target organs such as bone, kidney and gut (Deluca *et al.*, 1990; Stern., 1990; Walter *et al.*, 1992). These hormones exert their principal biological effects by activating specific intracellular receptors located in the cell nucleus. The activated receptors initiate the transcription of specific mRNAs which modulate the synthesis of the functional proteins that regulate calcium homeostasis such as calmodulin (Drittanti *et al.*, 1993), osteocalcin (Ikeda *et al.*, 1992; Antoniazzi *et al.*, 1993), and an unknown factor which is required for the activation of osteoclast (McSheehy *et al.*, 1987). These actions of vitamin D$_3$ and its metabolites usually occur after a long period and are known as genomic effects. As discussed in Chapter I, vitamin D$_3$ and its metabolites also have rapid effects which have been observed in many types of cells. Since this rapid effect takes place within sec or min, it is unlikely to involve protein synthesis (Wehile, 1994). Therefore, it was described as the "nongenomic effect" by some authors (Norman *et al.*, 1991). The nongenomic effects of vitamin D$_3$ and its metabolites are mainly involved in the regulation of $[Ca^{2+}]_i$, including its release from

intracellular calcium stores and the influx of calcium via the voltage dependent calcium channels (Norman, 1993). However, previous studies have focused on the effect of $1,25D_3$, the major active metabolite of vitamin D_3. The $24,25D_3$, another metabolite of vitamin D_3 with a high serum concentration, has received little attention with respect to its nongenomic action (Civitelli and Avioli, 1994). Therefore, the present study was undertaken to examine whether $24,25D_3$ also had nongenomic action on the voltage-dependent calcium channels in UMR-106 cells. It was found that $24,25D_3$ had a biphasic effect on the L type calcium channel but had no effect on the T type calcium channel. This biphasic effect of $24,25D_3$ was likely mediated through membrane receptors since it could be reversed by wash-out. The observation that the biphasic effect of $24,25D_3$ was inhibited or enhanced by nifedipine and Bay K 8644, respectively, confirmed that the effect of $24,25D_3$ was specific for the L type calcium channel.

4.2. Experimental Design

The whole cell version of the patch clamp technique and intracellular calcium measurement were used in these experiments to investigate the effect of $24,25D_3$ on the calcium channel. Wash-out was carried out in some experiments in order to evaluate whether the effect of $24,25D_3$ was secondary to a membrane-associated process. The time-courses of the effects of different concentrations of $24,25D_3$ on the L type calcium channel were also determined in some cells.

4.3. Results

4.3.1. The effect of 24,25D_3 on the L type calcium channel

To investigate the effects of 24,25D_3 on the voltage-dependent calcium channel, the T and L type calcium channels were examined using the protocols described in Chapter III. After the currents reached steady state and the control currents were obtained, different concentrations of 24,25D_3 or other agents were applied in the bath solution. The inward calcium currents were recorded at every 5 min after the application of the agents. The current amplitudes recorded at the time when the effects on the calcium current induced by the agents reached maximum were used for statistical comparisons.

24,25D_3 had a biphasic effect on the L type calcium channel. Fig. IV-1 shows that 24,25D_3 increased the L type calcium current in a concentration range between $5x10^{-10}$M and $1x10^{-7}$M. The maximum increase occurred at a concentration of $1x10^{-8}$M. At this concentration, the peak inward current was increased by $49\pm11\%$ ($n=11$, $p<0.05$). In contrast, concentrations greater than $1x10^{-7}$M had an inhibitory effect on the L type calcium current. At a concentration of $1x10^{-5}$M, 24,25D_3 decreased the peak current to $45\pm7\%$ ($n=9$, $p<0.05$) of the control current. Fig. IV-2 shows the enhancing effect of a low concentration of 24,25D_3 on the L type calcium current in a UMR-106 cell. In this cell, $1x10^{-8}$M of 24,25D_3 increased the peak current from 106 pA to 238 pA. The effect was more evident at the peak of the I-V relationship curve. However, the potential at which the inward current was activated and the peak inward current occurred was not

affected by this concentration of $24,25D_3$. In some cells, Bay K 8644 ($1x10^{-6}M$) was given after the effect caused by low concentration of $24,25D_3$ stabilized. The addition of Bay K 8644 further increased the current amplitude (data not shown). The inhibitory effect on the L type calcium current induced by a high concentration ($1x10^{-5}M$) of $24,25D_3$ is shown in Fig. IV-3. Even though the peak inward current was decreased from 72 pA to 33 pA by this concentration of $24,25D_3$, the I-V curve did not shift along the voltage axis. Similar to the effects of a low concentration of $24,25D_3$, the reduced current caused by a high concentration of $24,25D_3$ was reversed by the addition of Bay K 8644 (data not shown), indicating that the biphasic effects of $24,25D_3$ were specific to the L type calcium channels. The current records shown in Fig. IV-2 and Fig. IV-3 also indicate that neither a low nor a high concentration of $24,25D_3$ alter the kinetics of inactivation of the L type calcium currents.

In contrast to the effects on the L type calcium channel current, $24,25D_3$ did not affect the T type calcium channel currents in UMR-106 cells (data not shown).

4.3.2. The effect of $24,25D_3$ on the change in intracellular calcium induced by KCl

To correlate the observations from patch clamp studies, the effect of $24,25D_3$ on the rise in intracellular Ca^{2+} concentration (Fura-2 as the Ca^{2+} indicator) induced by KCl was examined in UMR-106 cells. KCl was used to depolarize the cells and this resulted in the opening of the voltage dependent calcium channels. The influx of calcium through the activated calcium channels resulted in an increase in $[Ca^{2+}]_i$. In these experiments, the cells were first stimulated by KCl (15 mM) and the increase in intracellular Ca^{2+} was

taken as the control. After washing with K^+ free solution, the cells were treated with different concentrations of $24,25D_3$ for 10 min followed by the subsequent addition of 20mM KCl. The increase in $[Ca^{2+}]_i$ caused by the second addition of KCl was taken as the effect of the $24,25D_3$ on $[Ca^{2+}]_i$.

The basal level of $[Ca^{2+}]_i$ in UMR-106 cells was around 70 ± 12 nM (n=8) (2mM Ca^{2+} in the bath solution). 20 mM KCl caused a rapid increase in $[Ca^{2+}]_i$ to 124 ± 20 nM (n=8). In the experiments presented in Fig. IV-4 and Fig. IV-5, the $24,25D_3$ at both $1x10^{-8}$M and $1x10^{-5}$M did not change the basal levels of $[Ca^{2+}]_i$, indicating that $24,25D_3$ had no effect on basal $[Ca^{2+}]_i$ in our preparation. However, pretreatment of these cells with $1x10^{-8}$M $24,25D_3$ potentiated the KCl-induced increase in $[Ca^{2+}]_i$ (Fig. IV-4). The summary of the results obtained from 4 experiments is presented in the bottom panel in the Figure. In contrast, the response of $[Ca^{2+}]_i$ to 20 mM KCl was attenuated by the pretreatment with $1x10^{-5}$M $24,25D_3$ (Fig. IV-5).

4.3.3. The effect of wash-out on the $24,25D_3$-induced change in L type calcium channel current

To examine the possibility that the biphasic effect of $24,25D_3$ on the L type calcium channel was a receptor-mediated event, wash-out (bath solution exchange) experiments were performed in cells after the effect of $24,25D_3$ on the L current had reached the steady state. Wash-out was performed using a set up which replaced the bath solution in 1 to 2 min without disturbing the recording.

Fig. IV-6 represents the results obtained from one representative cell. In this

experiment, the cell was first treated with a high concentration ($1x10^{-5}M$) of $24,25D_3$. When the inhibitory effect on the L current reached its maximum, wash-out was performed. After the current recovered to the control level, a low concentration ($1x10^{-9}M$) of $24,25D_3$ was added to the bath. This resulted in an increase in the L type current. After the increase in current induced by the low concentration of $24,25D_3$ reached the steady state, a second wash-out was performed. As shown in the Fig. IV-6, both the inhibitory and enhancing effects of two concentrations of $24,25D_3$ on the currents were reversible. However, a long time (> 10 min) was required before the current recovered to the control level. The currents started to recover a few min after the completion of the wash-out. In a group of 4 cells, the recovering time was 11 ± 3 min for the currents which had been increased by $1x10^{-9}M$ $24,25D_3$. In 4 other cells, the recovery time was 13 ± 3 min for the currents which had been decreased by $1x10^{-5}M$ $24,25D_3$. Fig. IV-7 and Fig. IV-8 show that the effect of two concentrations of $24,25D_3$ ($1x10^{-5}M$ and $1x10^{-8}M$) were reversed by wash-out in 4 cells in each group.

4.3.4. The comparison of the effects of $24,25D_3$ and $1,25D_3$ on the L type calcium channel current

The effect of a high concentration of $1,25D_3$ ($1x10^{-6}M$) on the L type calcium channel current was compared with that of $24,25D_3$ using the same experimental procedure. Fig. IV-9 shows the effects of $1,25D_3$ and wash-out on the L type calcium current in one cell. In this cell, $1,25D_3$ ($1x10^{-6}M$) increased the peak current amplitude from 165 pA to 220 pA and this increase was reversed by wash-out. Fig. IV-10 is the

summary of 5 similar experiments. At a concentration of $1x10^{-6}M$, $1,25D_3$ increased the L type calcium currents to $128\pm6\%$ ($n=5$, $P<0.05$). The time course of the effect of $1,25D_3$ and the effect of wash-out were measured in a group of 3 cells. Fig. IV-11 shows that the maximum effect of $1,25D_3$ occurred after 15 min. The increase in the current amplitude was reversed by wash-out and the recovery time for the current was 10 ± 3 min (data not shown).

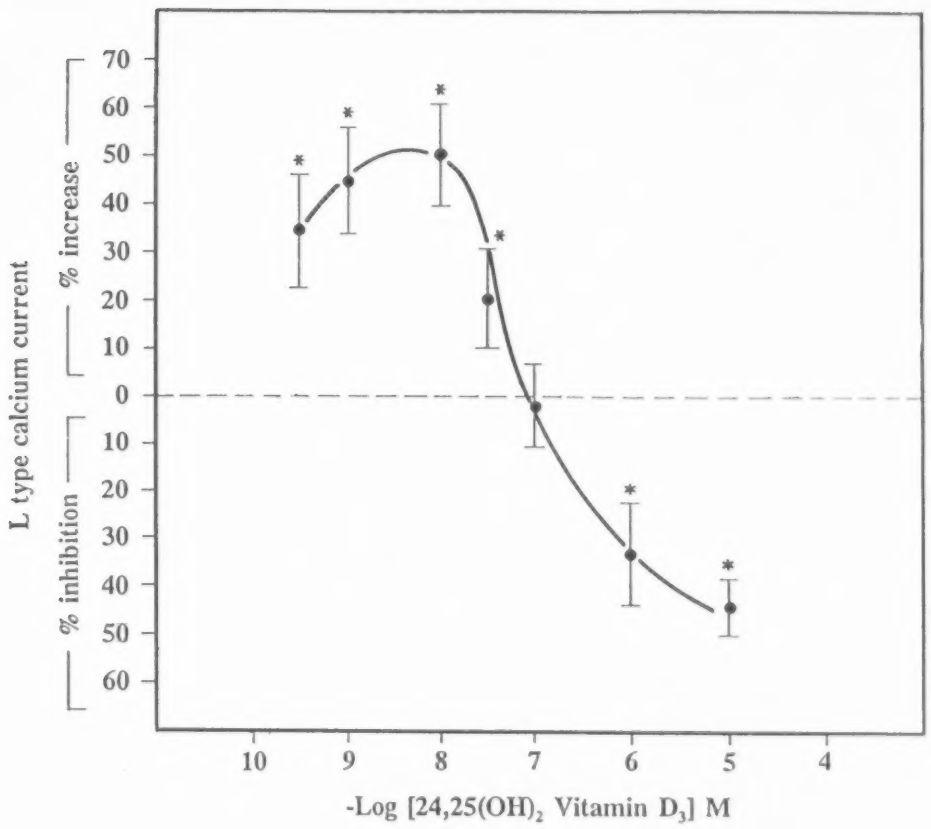

Fig. IV-1. The biphasic effect of $24,25D_3$ on the L type calcium channel currents in UMR-106 cells. The $24,25D_3$ had a biphasic effect on the L type calcium channel currents in UMR-106 cells. In the concentration range between $5x10^{-10}$M and $1x10^{-7}$M, $24,25D_3$ increased the amplitude of the L type calcium channel current. The maximum effect occurred at a concentration of $1x10^{-8}$M. At this concentration, $24,25D_3$ increased the current amplitude by $49\pm11\%$ ($n=11$, *, $p<0.05$). However, at the concentrations higher than $1x10^{-7}$, $24,25D_3$ decreased the L current amplitude. At a concentration of $1x10^{-5}$M, $24,25D_3$ inhibited the current amplitude by $45\pm7\%$ ($n=9$, *, $p<0.05$).

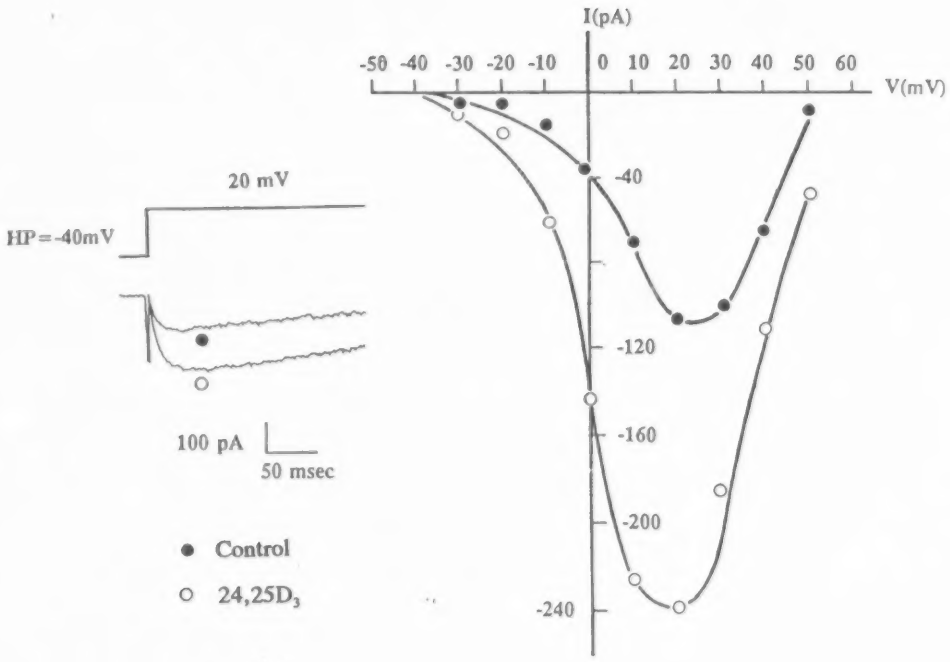

Fig. IV-2. The effect of a low concentration of $24,25D_3$ on the L type calcium channel current in a UMR-106 cell. The current records shown at left were elicited by depolarizing the membrane potential to 20 mV from a holding potential of -40 mV. The I-V relationships are shown on the right. The current records were obtained before the application (filled circles) and after the application (open circles) of $1x10^{-8}$M of $24,25D_3$. In this cell, the peak current amplitude was increased from 106 pA to 238 pA by $24,25D_3$. However, the potential at which the peak inward current was activated was not affected by $24,25D_3$. The inactivation rate of the current was also not affected by $24,25D_3$.

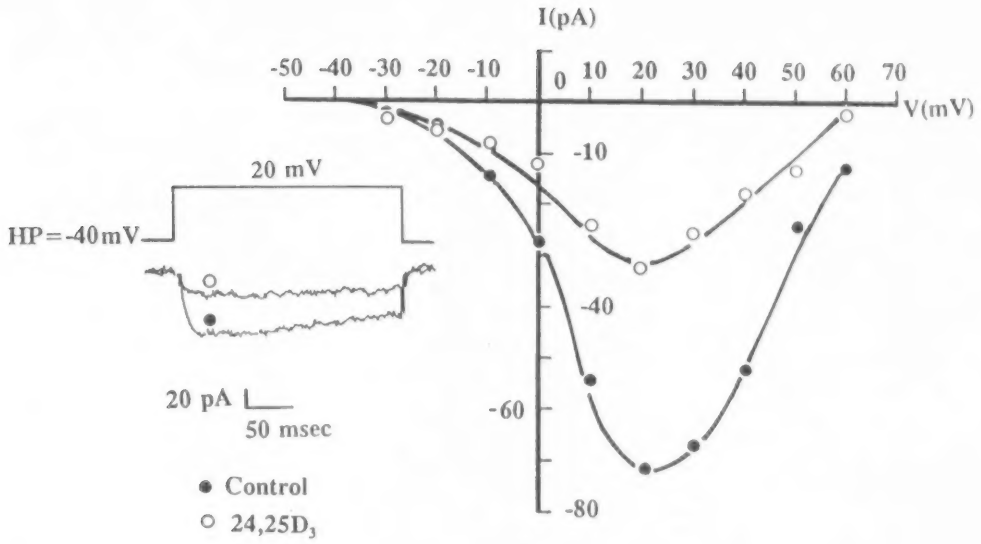

Fig. IV-3. The inhibitory effect of a high concentration of $24,25D_3$ on the L type calcium channel current in a UMR-106 cell. The current records shown at left were elicited by depolarizing the cell to 20 mV from a holding potential of -40 mV. The control current (filled circles) and the current after the addition of $1x10^{-5}M$ $24,25D_3$ (open circles) are shown. In this cell, the peak current amplitude was decreased from 72 pA to 33 pA by $24,25D_3$. The potential at which the peak current occurred was not shifted by $24,25D_3$.

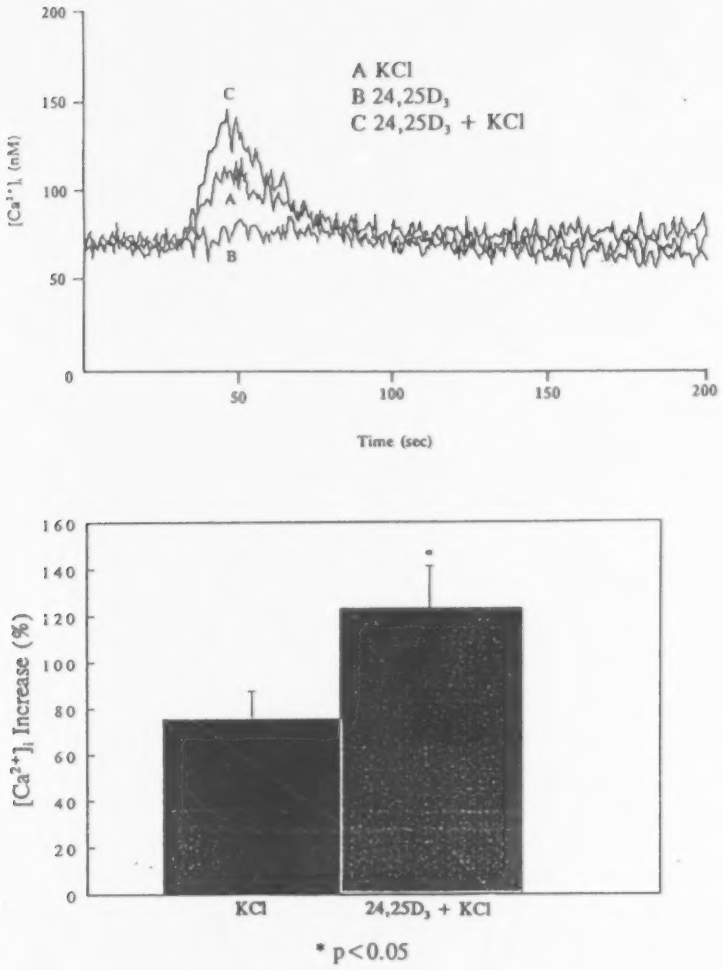

Fig. IV-4. The effect of a low concentration $24,25D_3$ on the intracellular calcium change induced by KCl in UMR-106 cells. The UMR-106 cells were grown on glass cover slips and loaded with Fura-2AM. Three intracellular calcium records are shown in the top panel. The cells were stimulated with KCl ($2x10^{-2}M$) (A). After three washs with K^+ free solution, the cells were then pretreated with $24,25D_3$ ($1x10^{-8}M$) (B) for 10 min and stimulated with KCl $2x10^{-2}M$ (C). In this experiment, KCl induced a transient increase in the intracellular calcium concentration. After pretreatment with $24,25D_3$, the response to KCl stimulation was potentiated. In 4 experiments, the basal level of $[Ca^{2+}]_i$ was 70 ± 12 nM. KCl ($2x10^{-2}M$) induced a 74 ± 12 nM ($n=4$) increase in intracellular calcium from the basal level. After the pretreatment with $24,25D_3$ ($1x10^{-8}M$), KCl caused an 122 ± 18 nM ($n=4$, $p<0.05$) increase in intracellular calcium from the basal level. This is shown in the bottom panel.

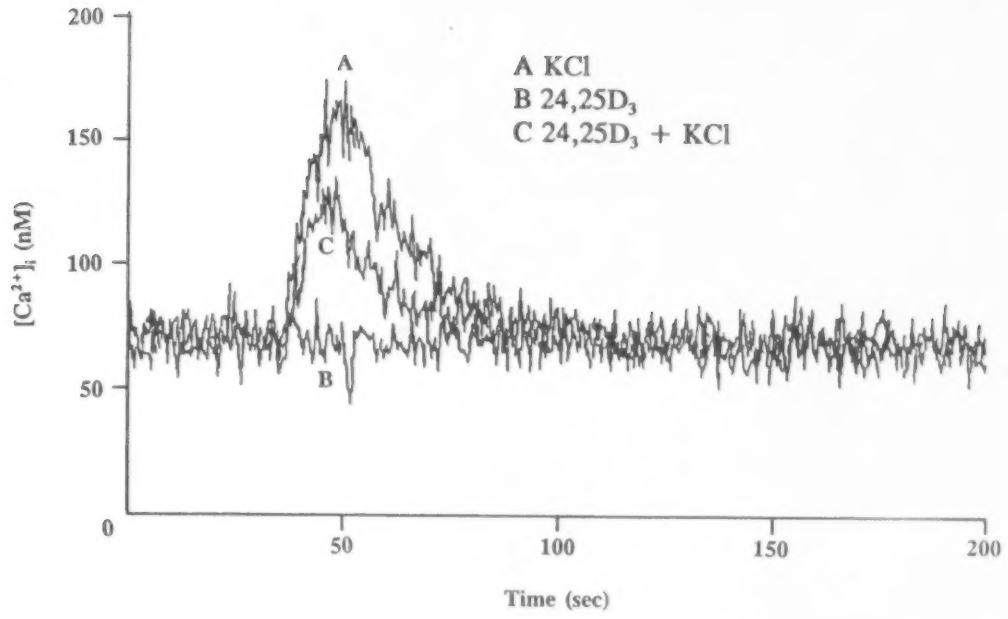

Fig. IV-5. The effects of a high concentration of 24,25D$_3$ on the intracellular calcium change induced by KCl in UMR-106 cells. The experimental procedure was the same as described in Fig. IV-4. In this experiment, in the presence of 24,25D$_3$ ($1x10^{-5}$M), the increase in the intracellular calcium induced by $2x10^{-2}$M KCl was decreased. The trace A represents the increase in intracellular calcium induced by KCl. The trace B represents the 24,25D$_3$ treatment, The trace C represents the intracellular calcium response to KCl after the pretreatment of 24,25D$_3$.

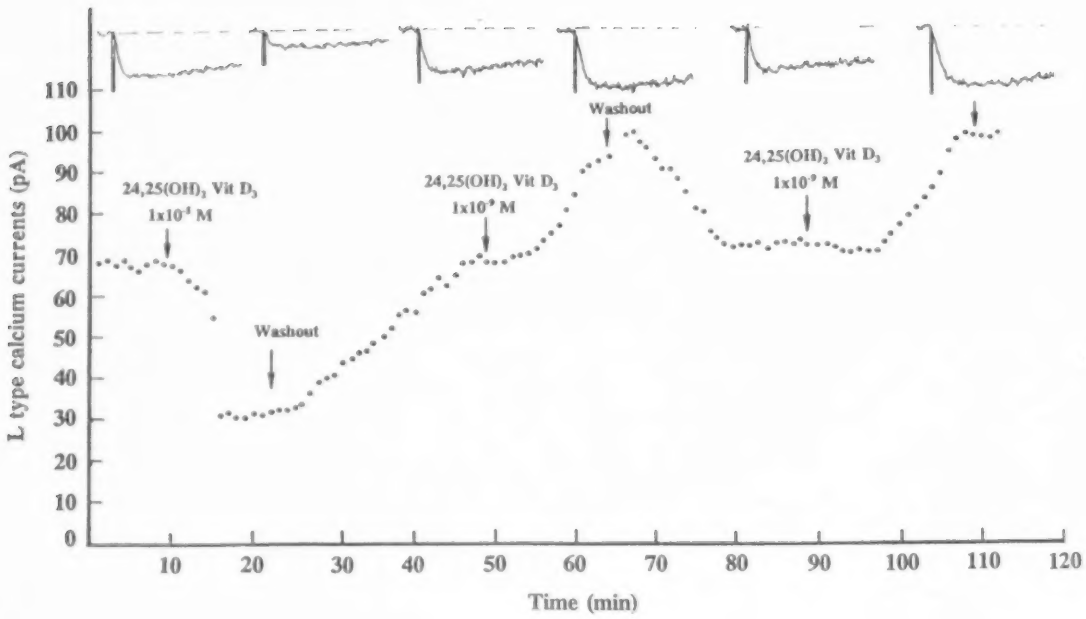

Fig. IV-6. The effect of wash-out on the biphasic effect of $24,25D_3$ on the L type calcium channel currents in a UMR-106 cell. The current records shown at the top of the Fig were obtained by depolarizing the membrane to 20 mV from a holding potential of -40 mV. The arrows below each current record indicate the times at which the corresponding currents were recorded. In this experiment, wash-out was performed with a system which could totally replace the bath solution in 1 to 2 min. The wash-out was done when the effect of the $24,25D_3$ reached maximum. The Fig. shows that the enhancing effect caused by a low concentration and the inhibitory effect caused by a high concentration of $24,25D_3$ could be reversed by wash-out.

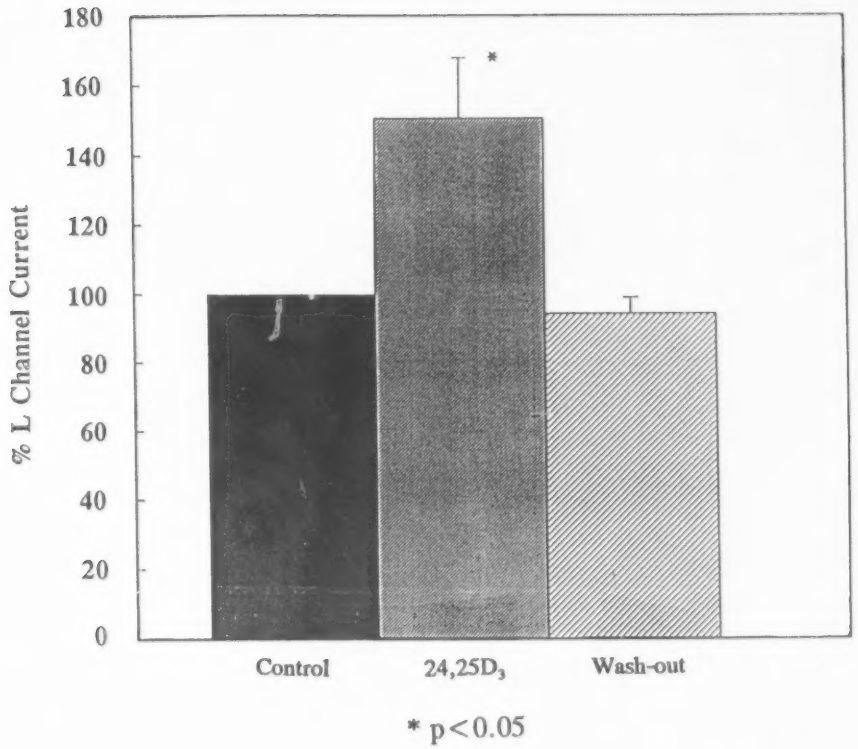

Fig. IV-7. The effect of wash-out on the effect of $24,25D_3$ on the L type calcium channel currents in UMR-106 cells. In a group of 4 cells, $24,25D_3$ ($1x10^{-9}M$) increased the peak current amplitudes to $148\pm16\%$. This increase in the current amplitude was reversed to $96\pm6\%$ of the control level by wash-out (*, $p<0.05$).

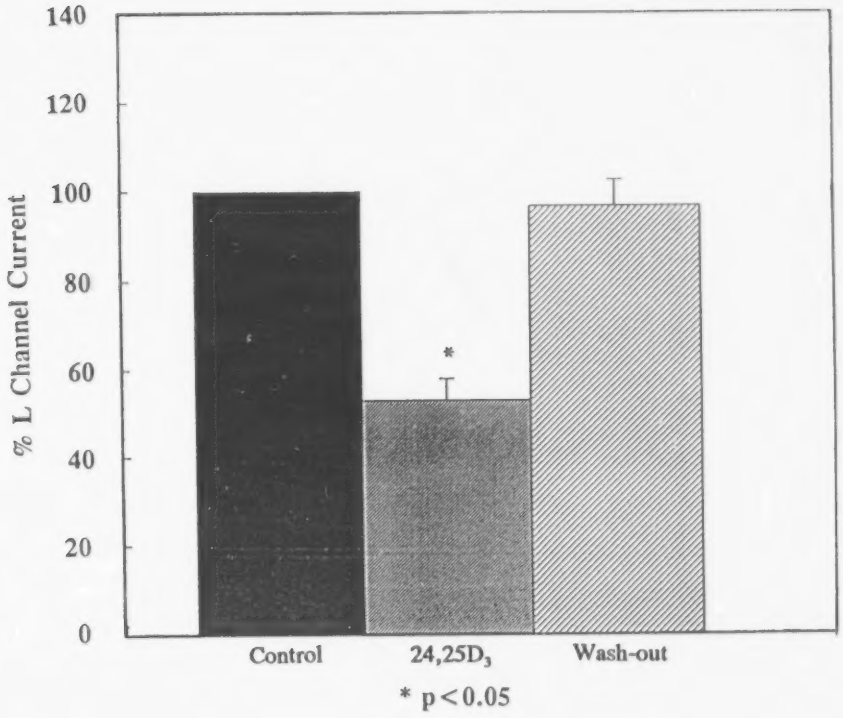

Fig. IV-8. The effect of wash-out on the inhibitory effect of $24,25D_3$ on the L type calcium channel current in UMR-106 cells. In a group of 4 cells, $24,25D_3$ ($1x10^{-5}M$) decreased the current amplitudes to $48\pm6\%$. This decrease in the current amplitude was reversed to $94\pm7\%$ of the control level by wash-out (*, $p<0.05$).

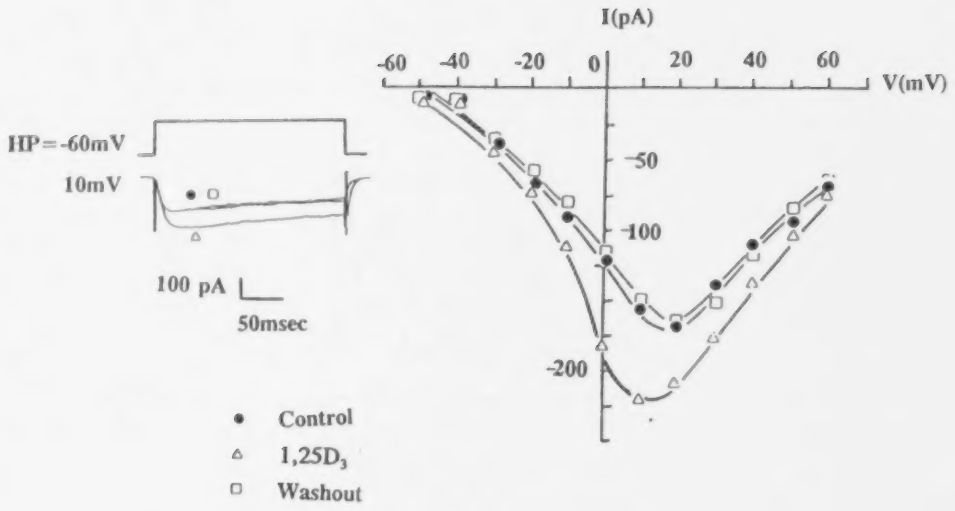

Fig. IV-9. The effect of $1,25D_3$ on the L type calcium channel current in a representative UMR-106 cell. The currents shown at left were activated by depolarizing the cell membrane to 10 mV from a holding potential of -40 mV. The control current (filled circles) was increased from 165 pA to 220 pA by the addition of $1x10^{-6}$M $1,25D_3$ (open triangles). The current increase was reversible by wash-out (open squares).

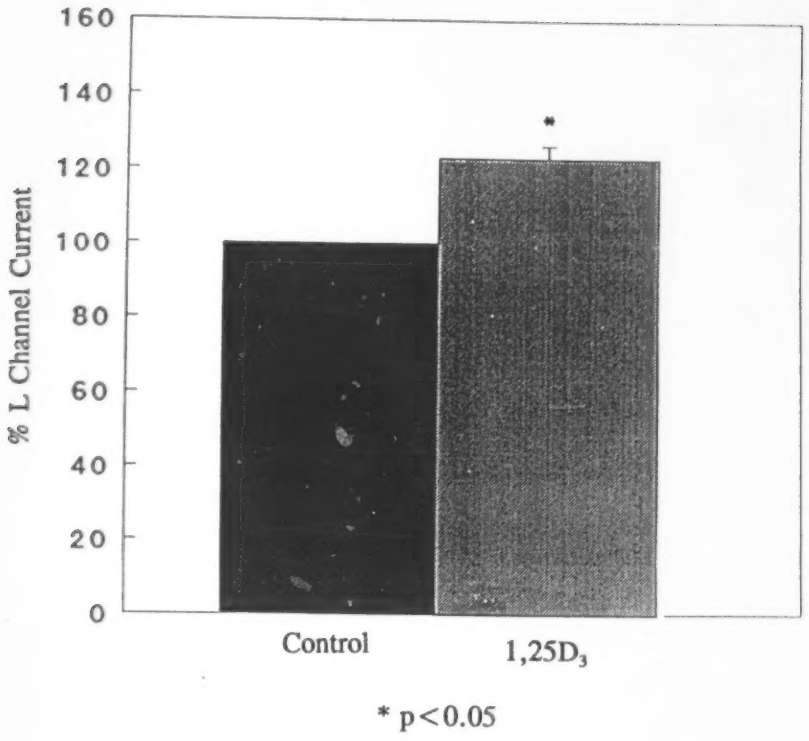

Fig. IV-10. The effects of a high concentrations of $1,25D_3$ on the L type calcium channel current in UMR-106 cells. At the concentration of $1x10^{-6}M$ of $1,25D_3$, the peak current amplitude was increased by $28\pm6\%$ (n=5, *, $p<0.05$).

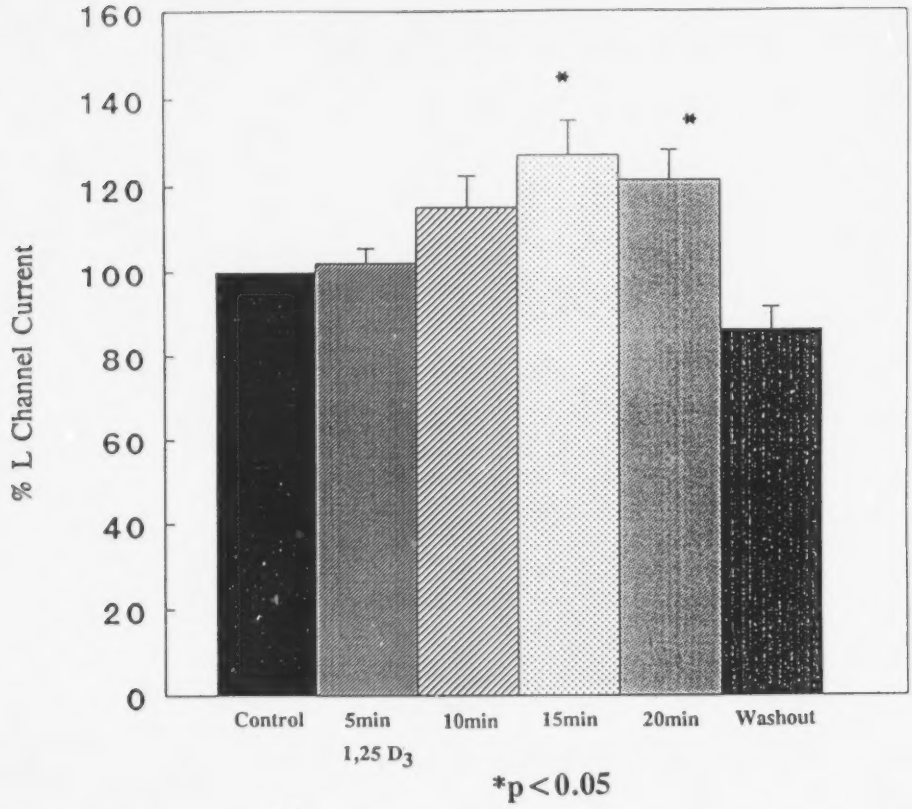

Fig. IV-11. The time course of the effect of $1,25D_3$ on the L type calcium channel current in UMR-106 cells. In a group of 3 cells, the maximal increase in the peak currents occurred at 10 to 15 min after the addition of $1,25D_3$ ($1x10^{-6}M$). The increase in the current amplitude was reversed by wash-out. (*, $p<0.05$).

4.4. Discussion

4.4.1. The significance of the nongenomic effects of $24,25D_3$ on the L type calcium channel

There is evidence that one of the major pathways employed by vitamin D_3 and its metabolites to generate biological responses is through their interaction with a cytosolic/nuclear receptor that mediates changes in gene transcription of specific proteins (Deluca, 1974; Holick, 1989; Civitelli and Avioli, 1994). However, it is apparent that some vitamin D_3 and its metabolites have the potential to generate biological responses via signal transduction pathways which are not dependent upon the regulation of gene transcription. This is known as the "nongenomic action" or "nongenomic effect" (Norman *et al.*, 1992; Weihal, 1994).

The $1,25D_3$ has been the main subject of investigation since it is the most biologically active form of vitamin D_3 (Civitelli *et al.*, 1990). However, much controversy exists with respect to the nongenomic effect of $24,25D_3$. Civitelli *et al.* (1990) reported that $1,25D_3$ and $25D_3$, but not $24,25D_3$, induced an immediate and rapid increase in $[Ca^{2+}]_i$ in ROS 17/2.8 cells. In agreement, Farach-Carson *et al.* (1991) showed that $24,25D_3$ did not cause an immediate transmembrane calcium influx at a concentration of $1x10^{-8}M$ in the same cell line. In contrast, Lieberherr (1987) showed that not only $1,25D_3$ and $25D_3$, but also $24,25D_3$, could induce an immediate increase in $[Ca^{2+}]_i$ in osteoblasts from neonatal mice. Furthermore, the increase in $[Ca^{2+}]_i$ induced

by $24,25D_3$ was much longer lasting than that caused by $1,25D_3$ and $25D_3$. In addition, it has also been reported that vascular perfusion of $24,25D_3$, at a concentration of $1.64x10^{-7}M$, increased the ^{45}Ca transport from duodenal lumen to the venous effluent in 10 min (Yoshimoto and Norman, 1986).

In addition to these observations, the sources of the increase in $[Ca^{2+}]_i$ induced by $24,25D_3$ also remains unclear. It was reported that release from the intracellular pool was the sole source for the increase in $[Ca^{2+}]_i$ induced by $24,25D_3$ in osteoblasts from neonatal mice (Lieberherr, 1987). However, the ^{45}Ca transport caused by $24,25D_3$ in intestinal cells was blocked by nifedipine, suggesting that the effect was dependent on extracellular calcium (Cancela *et al.*, 1988).

In this study, it was demonstrated that $24,25D_3$ had a concentration-dependent and biphasic effect on the L channel currents. However, $24,25D_3$ did not induce a significant change in the basal $[Ca^{2+}]_i$. This is the first documented nongenomic effect of $24,25D_3$ on the L type calcium channel in UMR-106 cells. The following observations support a nongenomic action of $24,25D_3$ on the voltage-dependent calcium channel current.

1) The biphasic effect of $24,25D_3$ on the L channel currents occurred quickly within 5 min after application and reached maximum within 15 min. This duration is too short for genomic action of vitamin D_3, an action which requires hours to develop (Weihal, 1994).

2) The observation that the effects of $24,25D_3$ could be reversed repeatedly by wash-out indicates that this quick action likely represents a membrane-associated process. If this effect is related to the synthesis or post-translational modification of functional

proteins by $24,25D_3$, it should not be reversible by wash-out since these functional proteins would be protected by the cell membrane.

4.4.2. The modulation of the L type calcium channel current by $24,25D_3$

Even though the mechanisms by which the voltage-dependent calcium channels are modulated have been extensively studied in many tissues, only a few papers address the regulation of voltage-dependent calcium channels by vitamin D_3 and its metabolites in bone cells (Civitelli and Avioli, 1994). In particular, the effects and the mechanisms of $24,25D_3$ on the voltage-dependent calcium channels remain largely unknown. This study describes several novel effects of $24,25D_3$ on the voltage-dependent calcium channel currents in UMR-106 cells.

1) The effect of $24,25D_3$ on the L type calcium channel may represent a membrane (receptor)-associated process. The receptors for vitamin D_3 and its metabolites are located in the cytosol and nucleus. A specific membrane receptor for $24,25D_3$ has not been found in bone cells (Civitelli and Avioli, 1994). However, the nongenomic effect of $24,25D_3$ appears to involve a membrane mediated process, since both the inhibitory and enhancing effects on the L type calcium current induced by $24,25D_3$ may be reversed by wash-out. As mentioned above, if the effects were brought about by functional proteins which were synthesized by genomic action of vitamin D_3, the effects should not be reversed by wash-out since these functional proteins, as well as the binding of vitamin D_3 to its nuclear receptors, are protected by the cell membrane and are, therefore, not reversible. In support of this speculation, Kim et al. (1994) reported that once $1,25D_3$

binds to the receptors in cytosol, the occupied vitamin D receptor could be recognized by a specific binding site on the endoplasmic reticulum and plasma membrane in ROS 17/2.8 cells. This binding recognizes the occupancy by $1,25D_3$ equal to that by steroid hormone analogues which exhibit only nongenomic activity but have little effect on the activation of the genomic (nuclear) activity of the vitamin D receptor (Kim and Hruska, 1994). Another important observation was made by Marx and Barsony (1994). They found that vitamin D stimulated the following rapid sequential changes in vitamin D receptors: clumping, centripetal alignment along microtubules, movement to the nuclear membrane, intranuclear uptake, and intranuclear aggregation. Cyclic GMP, an important second messenger, was found to accumulate around the clumped vitamin D receptors during this reorganization process and was quickly compartmentalized (Marx and Barsony, 1994). It is possible that $24,25D_3$ binds to vitamin D receptor in the cytosol after diffusing into the cell. The occupied vitamin D receptors bind to the binding site in the plasma membrane. This occupied vitamin D receptor-membrane binding site may in turn activate multiple second messenger system which modulates the voltage-dependent calcium channels (detailed discussion in Chapter V).

4.4.3. Correlation between *in vivo* and *in vitro* studies

The serum concentration of $24,25D_3$ under physiological condition is higher than that of $1,25D_3$ (Civitelli and Avioli, 1994). The high concentration of $24,25D_3$ in the serum was once considered as a metabolite of vitamin D_3 that was biologically inert (Henry and Norman, 1992). The demonstration of the nongenomic effect of $24,25D_3$ on

calcium channels suggests that $24,25D_3$ is an active metabolite of vitamin D_3 and may play an important role in regulating the function of bone cells. The concentration range for the enhancing effect on the L channel was between $1x10^{-10}M$ and $1x10^{-8}M$, similar to the physiological concentration of $24,25D_3$ in serum ($2.5x10^{-9}M$ to $1x10^{-8}M$) (Holick, 1989). Although the normal concentration of $24,25D_3$ in serum is 20 to 150 times higher than that of $1,25D_3$, and since the binding affinity of $1,25D_3$ to vitamin D receptors is a thousand times higher than that of $24,25D_3$ (Holick, 1989), under normal conditions, the effect of $24,25D_3$ on L channel in this concentration range may be overridden by the effect of $1,25D_3$. However, when the concentration of $24,25D_3$ is raised to very high levels, it may override the effects of $1,25D_3$ and result in the inhibition of the L type calcium channel. Our finding of the biphasic effect of $24,25D_3$ on calcium channel could also be of pharmacological importance. In support of this is the observation that some actions of $1,25D_3$ on bone can be antagonized by high concentrations of $24,25D_3$. It has been found that a 1000 times higher concentration of $24,25D_3$ (10-20 ng/ml) completely inhibits the ^{45}Ca-releasing effect of $1,25D_3$ (10-20 pg/ml) (Mahgoub, 1981). Furthermore, the suppressive effect of $1,25D_3$ on intracellular exchangeable calcium and on proline incorporation is antagonized by higher concentrations of $24,25D_3$ in bone cells from rachitic chicks (Kriajev and Edelstein, 1994). In contrast, it has been found that $24,25D_3$ used in an equivalent dose not influence the intestinal effects of $1,25D_3$ (Rubinger *et al.*, 1991).

It is well known that many cellular activities are controlled or regulated by $[Ca^{2+}]_i$. The modulation of voltage-dependent calcium channels is one crucial mechanism

by which $[Ca^{2+}]_i$ is regulated. Our finding that $24,25D_3$ inhibits L type calcium channel in high concentrations may, at least partly, explain the above observations.

Correlation between structure and the function of vitamin D_3 metabolites has been noted for some time (Norman *et al.*, 1992). The molecular structures of vitamin D_3 and its metabolites are very similar. Small changes in the structure, especially at the position of the hydroxylation, can result in a change in the function of the metabolite (Evans *et al.*, 1990; Harada *et al.*, 1992). This change in function caused by a small change in structure has been observed in many hormones and steroids. For example, PTH(3-34) lost the whole function of PTH and became a PTH antagonist though only two amino acids at N' terminal were deleted (Rosenblatt, 1986; Wang *et al.*, 1990).

Some different actions of vitamin D_3 and its metabolites have also been brought to attention. Civitelli *et al.* (1990) reported that, in ROS 17/2.8 cells, $1,25D_3$ induced both Ca^{2+} influx and intracellular Ca^{2+} release; $25D_3$ only induced Ca^{2+} influx while $1\alpha,D_3$ and $24,25D_3$ had no effect on intracellular Ca^{2+} release. Caffrey and Farach-Carson (1989) also demonstrated that the L type calcium channel could be opened by $1,25D_3$ but not by $24,25D_3$. Therefore, it was postulated that hydroxylation at position 25 is required for the voltage-mediated Ca^{2+} influx. While hydroxylation in positions 1α and 25 is necessary for intracellular Ca^{2+} release, hydroxylation at position 24 appears to antagonize the action of the hydroxylation at position 25 on plasma membrane Ca^{2+} permeability (Civitelli *et al.*, 1990). In agreement with this hypothesis, $1,25D_3$ was found to increase the L channel current while high concentrations of $24,25D_3$ inhibited this current. However, similar to $1,25D_3$, low concentrations of $24,25D_3$ also increased the

L channel current in this study. Therefore, it is possible that in addition to the position of hydroxylation, the number of hydroxyl groups present may also determine the action of the vitamin D_3 metabolites.

4.4.4. The biphasic effect of 24,25D_3 on the L channel current and membrane fluidity.

An accepted membrane action of steroid hormones is their ability to change membrane fluidity. It has been reported that steroids can change the composition of the phospholipids in the cell membrane, which, in turn, changes the membrane fluidity (Swain *et al.*, 1993). As a consequent of the changes in membrane fluidity, the membrane permeability for Ca^{2+} may increase. However, it is unlikely that this accounts for the biphasic effect of 24,25D_3 on the L channel current. If the L channel current effect of both vitamin D metabolites is due to their action on membrane fluidity, the effect of 24,25D_3 should be similar to that of 1,25D_3 since their structures are similar . However, at a concentration of $1x10^{-6}$M, 1,25D_3 increased the L channel current while 24,25D_3 inhibited this current. This suggests that the effects of 24,25D_3 on L channel current are unlikely to be due to altered membrane fluidity alone. Furthermore, as discussed in the following chapter, the biphasic effect of 24,25D_3 is mediated by protein kinase A and protein kinase C and is unlikely to be a result of changes in membrane permeability alone.

Chapter V

The modulation of the effect of 24,25D_3 on the L type calcium channel current by second messenger systems

5.1. Introduction

It is well documented that the voltage-dependent calcium channel can be modulated by intracellular second messenger systems which involve protein kinases (Curtis and Catterall, 1985; Rane and Dunlap, 1986; Vivaudou *et al.*, 1988; Marchetti and Brown, 1988; Braha *et al.*, 1993; Ishikawa *et al.*, 1993). These protein kinases, once activated by the ligand-receptor coupling, phosphorylate calcium channel proteins resulting in an alteration in the gating of calcium channels. Many peptide hormones and neurotransmitters have been demonstrated to modulate the activity of calcium channels through second messenger systems. Wang *et al.* (1991) reported that PTH(1-34) inhibited the L type calcium channel current and that this inhibition was mediated by cAMP/PKA in smooth muscle cells from rat tail artery. In skeletal muscle cells, Chang *et al.* (1991) showed that the activity of DHP-sensitive calcium channels was increased by PKA and PKC. 4β-phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, has been shown to increase the calcium channel current in neonatal rat cardiac myocytes and PKC also mediates the effects of angiotensin II on this current (Dosemeci *et al.*, 1987).

Vitamin D_3 and its metabolites have been demonstrated to stimulate multiple signalling systems in different cells: PI turnover and PKC activation in osteoblasts and

skeletal muscle (Baran and Kelly, 1987; Baran *et al.*, 1992; van Leeuwen *et al.*, 1990, 1992; Grosse *et al.*, 1993), cGMP accumulation in fibroblasts (Barsony and Marx, 1991) and cAMP/PKA activation in bone cells (Kubota *et al.*, 1985). Nongenomic actions of vitamin D_3 and its metabolites on bone have been shown to be regulated by second messenger systems. Civitelli *et al.* (1990) demonstrated that the rapid increase in $[Ca^{2+}]_i$ induced by $1,25D_3$ in ROS 17/2.8 cells was accompanied by an increase in the production of IP_3. Furthermore, the addition of a second dose of $1,25D_3$ within 5 min resulted in a higher increase in $[Ca^{2+}]_i$, suggesting that PKC may mediate the effect of $1,25D_3$ on $[Ca^{2+}]_i$. The involvement of PKC in the action of $1,25D_3$ in bone had also been observed (Van Leeuwen *et al.*, 1992). In another report, PKC has been shown to modulate the rapid increase in $[Ca^{2+}]_i$ in skeletal muscle cells induced by $1,25D_3$ (de Boland *et al.*, 1993). These observations indicate the involvement of multiple intracellular second messenger systems in the nongenomic actions of vitamin D_3 and its metabolites.

In chapter IV, $24,25D_3$ was shown to have a rapid and biphasic effect on the L calcium current in UMR-106 cells. This effect of $24,25D_3$ likely represents a membrane-associated process and may involve the activation of multiple signalling systems. To investigate this possibility, experiments were undertaken to examine the role of PKA, PKC and G proteins in the effect of $24,25D_3$ on the L type calcium channel current. It was found that multiple signalling pathways were involved in the effect of $24,25D_3$ on the L type calcium channel currents in UMR-106 cells. At low concentrations, the action of $24,25D_3$ was dependent on the PKA pathway. In contrast, at high concentrations, both PKA and PKC pathways were involved with PKC playing a dominant role.

5.2. Experimental Design

The whole cell version of the patch clamp technique and intracellular cAMP accumulation measurement were employed in these experiments as described in Chapter II. To determine the involvement of second messengers in the effect of $24,25D_3$ on the L type calcium channel, activators and inhibitors of PKA, PKC and G proteins were used in the following experiments.

5.3. Results

5.3.1. The effect of 8-bromo-cAMP on the L type calcium channel currents in UMR-106 cells

To investigate the involvement of the PKA signal pathway, the effect of extracellular application of 8-bromo-cAMP, a PKA activator, on the L type calcium channel was examined. As shown in the Fig.V-1, it was found that 8-bromo-cAMP, at concentrations ranging from $1x10^{-7}M$ to $1x10^{-5}M$, increased the L type calcium channel current and this increase was concentration-dependent. The maximal increase in the amplitude of the current occurred at a concentration of $5x10^{-5}M$. At this concentration, 8-bromo-cAMP increased the current amplitude by $122\pm34\%$ ($n=5$, $p<0.05$) when compared to the control level. However, at a concentration of $1x10^{-5}M$, the enhancing effect on the current amplitude was only $75\pm20\%$ ($n=5$, $p<0.05$) which was less than that of $5x10^{-6}M$.

5.3.2. The interaction between 8-bromo-cAMP and 24,25D_3 on the L type calcium channel current

In this experiment, cells were treated with $1x10^{-5}$M of 8-bromo-cAMP at the beginning. When the effect of 8-bromo-cAMP on the L type calcium channel reached steady state, 24,25D_3 ($1x10^{-8}$M) was added. The results in Fig.V-2 show that 8-bromo-cAMP ($1x10^{-5}$ M) increased the peak current amplitude from 120 pA to 320 pA. The peak of the I-V curve was also shifted toward more negative direction (from 30 mV to 10 mV). The subsequent addition of 24,25D_3 ($1x10^{-8}$M) did not cause a further increase in the current amplitude and shift of the peak of the I-V curve; instead, it decreased the peak current amplitude from 320 pA to 250 pA, indicating that the effect of 24,25D_3 at a low concentration on the L type calcium channel currents could be abolished with pretreatment of 8-bromo-cAMP. This suggests that the effect of a low concentration of 24,25D_3 on the L type calcium channel current may be regulated by the cAMP/PKA pathway.

5.3.4. The intracellular application of Rp-cAMPs on the effect of 24,25D_3 on the L type calcium channel current

Intracellular application of Rp-cAMPs, a specific inhibitor of PKA, was used to examine whether the inhibition of PKA abolished the effect of a low concentration of 24,25D_3 on the L type calcium channel current.

In these experiments, Rp-cAMPs ($1x10^{-7}$M) was added into the pipette solution. After the whole cell configuration was established, a 10-min period time was used to

allow the Rp-cAMPs to diffuse into the cell and a control current was recorded. $24,25D_3$ was then applied. One representative result of these experiments on a cell is shown in Fig.V-3. It was found that after intracellular perfusion with Rp-cAMPs ($1x10^{-7}M$), the subsequent application of a low concentration of $24,25D_3$ ($1x10^{-8}M$) did not cause an increase in the current amplitude. This further confirmed that effect of low concentration of $24,25D_3$ on the L type calcium channel current was PKA dependent.

The effects of a medium and high concentrations of $24,25D_3$ were also tested with the same experimental protocol. The result is shown in Fig.V-4. In this cell, with the intracellular application of Rp-cAMPs, a higher concentration of $24,25D_3$ ($1x10^{-7}M$), which had no effect on the L type calcium current with the normal pipette solution (see Chapter VI), decreased the peak current amplitude from 43 pA to 32 pA (about 26%). With a subsequent addition of a higher concentration of $24,25D_3$ ($1x10^{-5}M$), the peak current amplitude was further decreased from 32 pA to 17 pA (about 59%). These results suggest that the inhibitory effects of $24,25D_3$ are PKA independent and that at higher concentrations, a second mechanism appears to mediate the inhibitory action of high concentrations of $24,25D_3$. This possibility will be described later. Fig.V-5. shows the effects of three concentrations of $24,25D_3$ on the L channel current in the presence and absence of Rp-cAMPs in the pipette solution.

5.3.5. The effects of $24,25D_3$ on cAMP accumulation

Since the above results indicated that the enhancing effect of $24,25D_3$ was mediated by a cAMP and PKA signal pathway, it might suggest that $24,25D_3$ has a direct

effect on the cAMP accumulation in UMR-106 cells. To examine this mechanism of $24,25D_3$ on cAMP/PKA signal pathway, the effects of $24,25D_3$ on the cAMP accumulation were evaluated. It was found that a 10-min treatment with $24,25D_3$ ($1x10^{-8}$M) did not significantly increase the intracellular cAMP accumulation. In the presence of a phosphodiesterase inhibitor, IBMX ($1x10^{-3}$M), $24,25D_3$ also did not increase cAMP accumulation. Fig.V-6 shows the results of cAMP accumulation stimulated by $1x10^{-8}$M $24,25D_3$ in the presence and absence of $1x10^{-3}$M IBMX.

5.3.6. The involvement of PKC on the action of $24,25D_3$ on the L type calcium channel currents

Since a second mechanism appears to mediate the inhibitory effect of higher concentrations of $24,25D_3$ on the L type calcium current and multiple PKC phosphorylation sites have been identified on the L type calcium channels, the possible involvement of PKC was examined, first, with extracellular application of a protein kinase inhibitor (staurosporine), Additional experiments were performed with two activators of PKC (PMA and OAG) and a specific PKC inhibitor (calphostin C).

5.3.6.1. The effect of staurosporine

Staurosporine is a potent but nonspecific protein kinase inhibitor. The experiment presented in Fig.V-7 shows that extracellular application of staurosporine caused a slight increase in the peak current amplitude from 148 pA to 172 pA in this cell. However, the subsequent addition of a high concentration of $24,25D_3$ ($1x10^{-5}$M) did not result in a further decrease in the current amplitude. Fig.V-8. shows the summary of the effect of

staurosporine and $24,25D_3$ on the peak current amplitude obtained from 7 cells. These results suggest that the inhibitory effect of a high concentration of $24,25D_3$ on the L type calcium channel current is protein kinase dependent.

5.3.6.2. The effects of PMA and OAG

The involvement of PKC was investigated using two PKC activators, PMA and OAG. The results indicated that activation of PKC resulted in inhibition at the L type calcium channel current and the inhibitory effect of higher concentrations of $24,25D_3$ on the L type calcium current was mediated by PKC. Fig.V-9. shows the results from a cell treated with PMA ($3x10^{-7}M$) for 10 min followed by treatment with a high concentration of $24,25D_3$ ($1x10^{-5}M$). In this cell, the peak current amplitude was decreased from 95 pA to 58 pA by PMA and the subsequent addition of $24,25D_3$ ($1x10^{-5}M$) had no effect on the current amplitude. A similar result was obtained with OAG. As shown in Fig.V-10, OAG ($1x10^{-5}M$) decreased the current amplitude from 79 pA to 52 pA in this cell. The subsequent addition of a high concentration of $24,25D_3$ ($1x10^{-5}M$) did not further decrease the current amplitude. The summary of the experiments using PMA is shown in Fig.V-11. It shows that $24,25D_3$ ($1x10^{-5}M$) decreased the current amplitude by 49.7% ($n=9$, $p<0.05$). Treatment with PMA ($3x10^{-7}M$) also decreased the peak current amplitude by $43\pm3\%$ ($n=5$. $p<0.05$). The subsequent addition of $24,25D_3$ ($1x10^{-5}M$) did not cause a further decrease in the peak current amplitude. In comparison, in a group of 5 cells, OAG ($1x10^{-6}M$) only decreased the peak current amplitude by $29\pm4\%$ ($n=5$, $p<0.05$) and the subsequent addition of $24,25D_3$ ($1x10^{-5}M$) reduced the peak current

amplitude further to 44±4% (n=5, $p<0.05$). The results are shown in Fig.V-12. These results suggest that the activation of PKC caused inhibition on the L type calcium channel currents and that the inhibitory effect of a high concentration of $24,25D_3$ on the L type calcium current was PKC dependent. Unlike the experiment with PMA, OAG ($1x10^{-6}M$) only reduced the current by 29% suggesting a submaximal activation of PKC; therefore, the subsequent addition of $24,25D_3$ ($1x10^{-5}M$) resulted in a further inhibition of the current amplitude.

5.3.6.3. The effect of calphostin C

The role of PKC on the effects of $24,25D_3$ on the L type calcium channel current was further evaluated using a specific PKC inhibitor, calphostin C. In these experiments, calphostin C ($1x10^{-6}M$) was added into the pipette solution. The cells were perfused with calphostin C for 10 min and then stimulated by different concentrations of $24,25D_3$. Fig.V-13 shows that, with the intracellular application of calphostin C, $24,25D_3$ ($1x10^{-7}M$), which had no effect on the L type calcium channel currents with normal pipette solution, increased the peak current amplitude from 68 pA to 86 pA. In the presence of calphostin C, the inhibitory effect caused by a high concentration of $24,25D_3$ ($1x10^{-5}M$) was also reversed resulting in a small increase in the current amplitude. These results suggest that high concentrations of $24,25D_3$ likely activate both cAMP/PKA and PKC pathways and the dominant effect on the current is mediated through PKC. Therefore, when PKC is blocked, an increase in the L type calcium channel current mediated through cAMP/PKA is observed.

To illustrate that both PKA and PKC are involved in the effect of by $24,25D_3$ on the L type calcium channel current, the results of two concentration of $24,25D_3$ ($1x10^{-7}M$ and $1x10^{-5}M$) on the L type calcium channel current in the presence and absence of calphostin C or Rp-cAMPs are summarized in Fig.V-14 and Fig.V-15. In a group of 4 cells, treatment with $24,25D_3$ ($1x10^{-7}M$) had no effect on the current amplitude with the normal pipette solution. However, the inclusion of calphostin C ($1x10^{-7}M$) in the pipette solution increased the current amplitude by $19\pm4\%$ ($n=4$, $p<0.05$), while the inclusion of Rp-cAMPs ($1x10^{-7}M$) in the pipette solution decreased the current amplitude by $28\pm9\%$ ($n=5$, $p<0.05$).(Fig. V-14). The $24,25D_3$ ($1x10^{-5}M$) decreased the peak current by $43\pm6\%$ ($n=11$, $p<0.05$) with the normal pipette solution. With calphostin C in the pipette solution, there was a slight increase in the current amplitude above control by $12\pm5\%$ ($n=4$, $p<0.05$); with Rp-cAMPs in the pipette solution, the current amplitude was decreased by $56\pm4\%$ ($n=4$, $p<0.05$) (Fig.V-15).

5.3.7. The involvement of G proteins in the effect of $24,25D_3$ on the L type calcium channel current

It is well known that G proteins are involved in the activation of PKA and PKC. Since PKA and PKC signal pathways were involved in the effect of $24,25D_3$ on the L type calcium channel current, it was reasonable to speculate that G proteins may also mediate the actions of $24,25D_3$. To examine this possibility, the effect of $24,25D_3$ on the L type calcium channel current was examined in the presence of GDPβs, a nonspecific G protein inhibitor. In this experiment, GDPβs ($1x10^{-3}M$) was added in the pipette

solution. After the cells were perfused with GDPβs-containing pipette solution for 10 min, the cells were stimulated with different concentrations of $24,25D_3$. The results obtained from a representative cell are shown in Fig.V-16. Both the enhancing effect of a low concentration ($1x10^{-8}M$) and the inhibitory effect of a high concentration ($1x10^{-5}M$) of $24,25D_3$ were abolished by the intracellular application of GDPβs. This suggests that the biphasic effect of $24,25D_3$ on the L type calcium channel is mediated by G proteins. Fig.V-17 shows the summary of the results obtained from 4 cells.

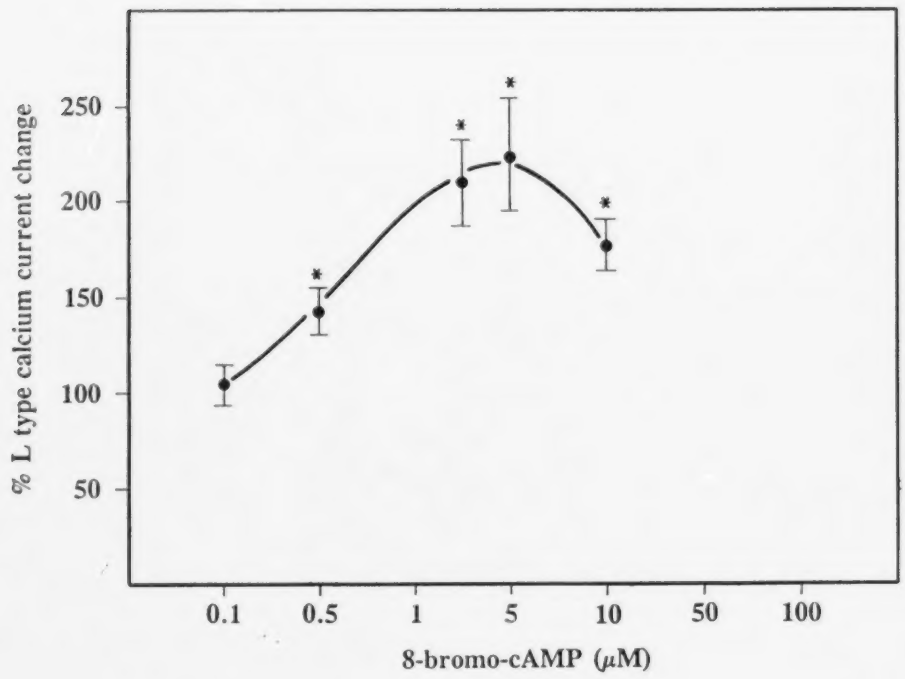

Fig. V-1. The effects of 8-bromo-cAMP on the L type calcium channel currents in UMR-106 cells. At all concentrations being tested, 8-bromo-cAMP increased the current amplitudes in UMR-106 cells. At a concentration of $5x10^{-6}$M, the peak current amplitude was increased by $122\pm34\%$ ($n=5$, *,$p<0.05$). However, at a higher concentration of $1x10^{-5}$M, 8-bromo-cAMP only increased the peak current amplitude by $75\pm20\%$ ($n=5$, *,$p<0.05$).

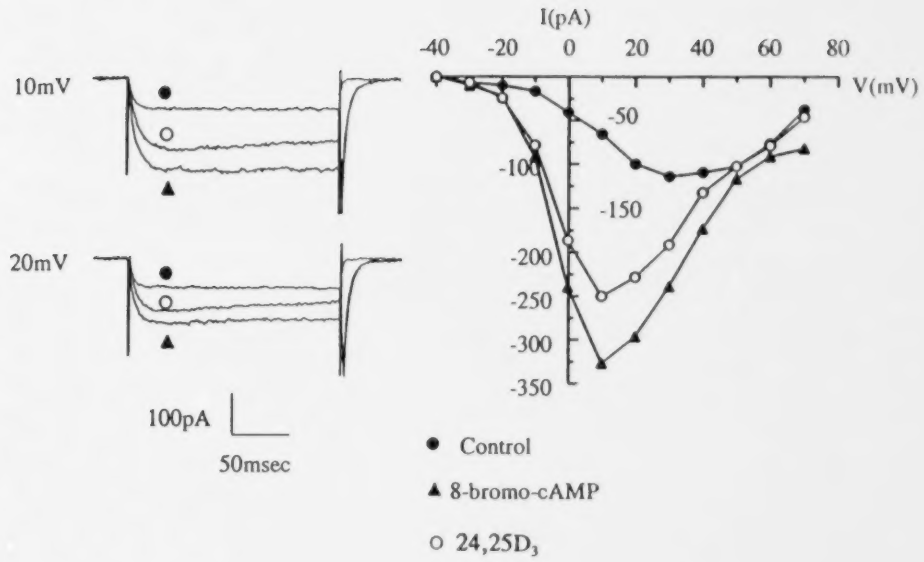

Fig. V-2. The effect of 8-bromo-cAMP and $24,25D_3$ on the L type calcium channel current in a UMR-106 cell. The current records shown at left were activated by depolarizing the cell membrane to 10 and 20 mV from a holding potential of -40 mV. In this cell, 8-bromo-cAMP $1x10^{-5}$M increased the control current (filled circles) from 120 pA to 320 pA (filled triangles) and shifted the peak of the I-V curve towards more negative potentials. The subsequent addition of $1x10^{-8}$M $24,25D_3$ did not cause a further increase in the current amplitude nor a shift in the I-V curve. Instead, $24,25D_3$ ($1x10^{-8}$ M) decreased the current amplitude from 320 pA to 250 pA (open circles).

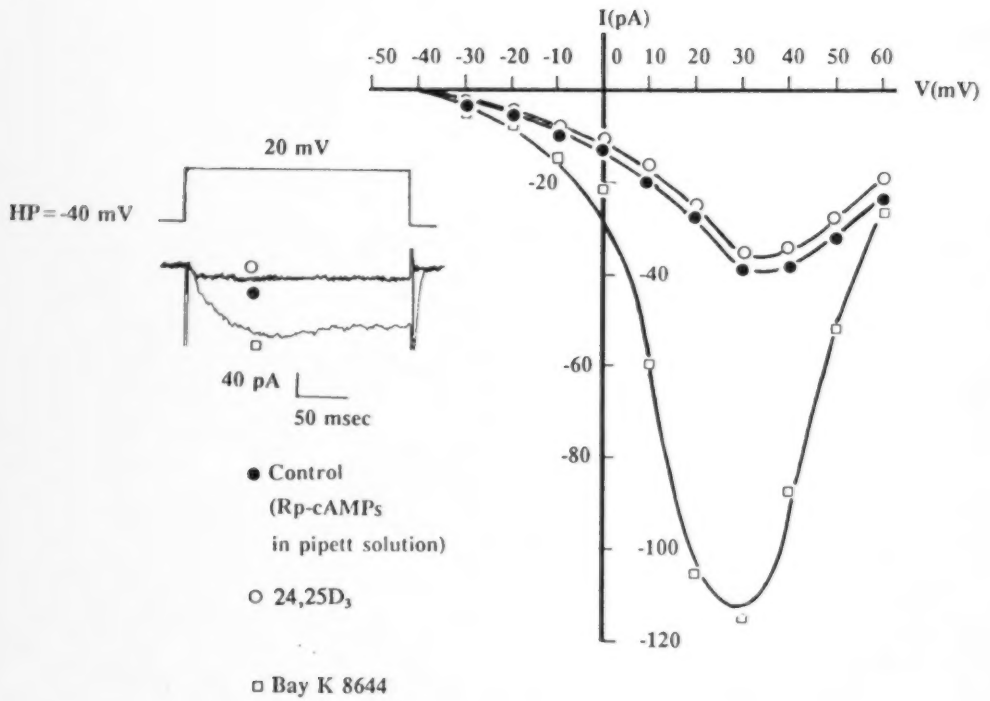

Fig. V-3. The effect of a low concentration of $24,25D_3$ on the L type calcium channel current in a UMR-106 cell perfused with Rp-cAMPs. In this experiment, the cell was perfused for 10 min with a pipette solution which contained $1x10^{-7}M$ of Rp-cAMPs (filled circles) and then treated with a low concentration of $24,25D_3$ ($1x10^{-8}M$) (open circles). In this cell, the effect of $24,25D_3$ $1x10^{-8}M$ on the L type calcium current was abolished by the intracellular perfusion of Rp-cAMPs ($1x10^{-7}M$). The current could be increased by Bay K 8644 after the cell was treated with Rp-cAMPs and $24,25D_3$, indicating that the lack of $24,25D_3$ effect was not due to cell death. The peak of the I-V relationship was not shifted by $24,25D_3$.

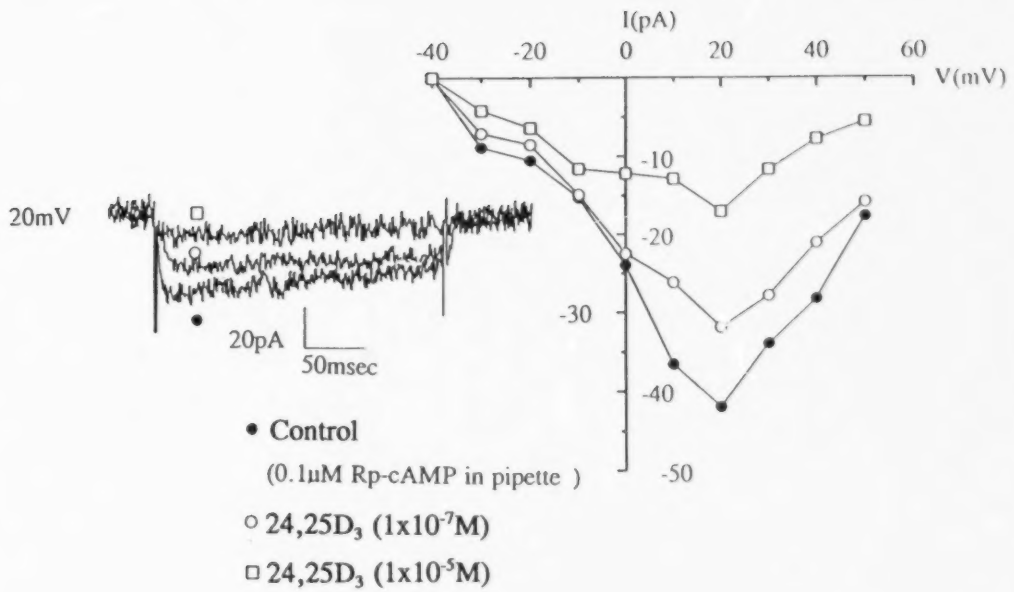

Fig. V-4. The effects of two concentrations of $24,25D_3$ on the L type calcium channel current in a UMR-106 cell perfused with Rp-cAMPs. The experimental procedure was the same as described in Fig.V-2. Perfused with $1x10^{-7}$M Rp-cAMPs, $24,25D_3$ at the concentrations of $1x10^{-7}$M and $1x10^{-5}$M decreased the peak current amplitude from 43 pA to 32 pA and from 32 pA to 17 pA, respectively.

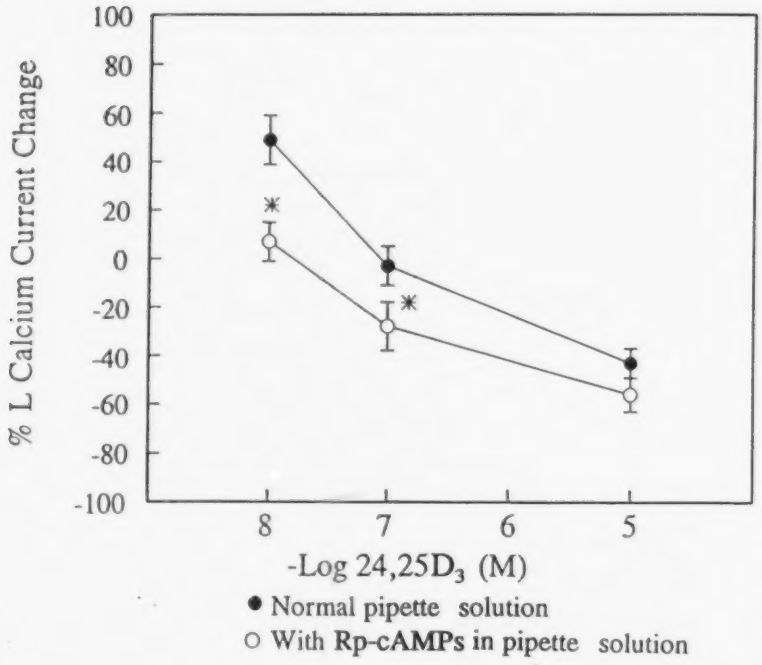

Fig. V-5. The comparison of the effects of three concentrations of $24,25D_3$ on the L type calcium channel currents in the presence and absence of intracellular Rp-cAMPs ($1x10^{-7}$M). The curve (filled circles) represents the effects of three concentrations of $24,25D_3$ on the L type calcium channel currents without the intracellular perfusion of Rp-cAMPs. The curve (open circles) represents the effects of three concentrations of $24,25D_3$ on the L type calcium channel currents with the intracellular perfusion of Rp-cAMPs. The inhibitory effects on the L type calcium channel currents caused by these concentrations of $24,25D_3$ became more potent in the presence of Rp-cAMPs. (*,$p<0.05$).

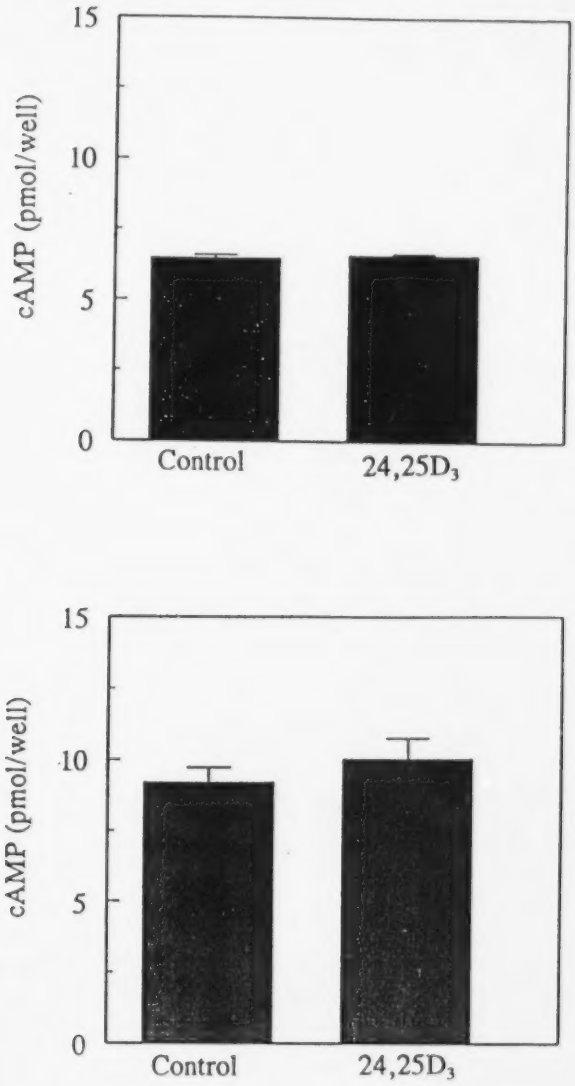

Fig. V-6. The effect of $24,25D_3$ on cAMP accumulation in UMR-106 cells. The top panel shows that $24,25D_3$ ($1x10^{-8}M$) did not significantly increase the intracellular cAMP accumulation. The bottom panel shows that in the presence of a phosphodiesterase inhibitor, IBMX ($1x10^{-3}M$), $24,25D_3$ also did not significantly increase the intracellular cAMP accumulation.

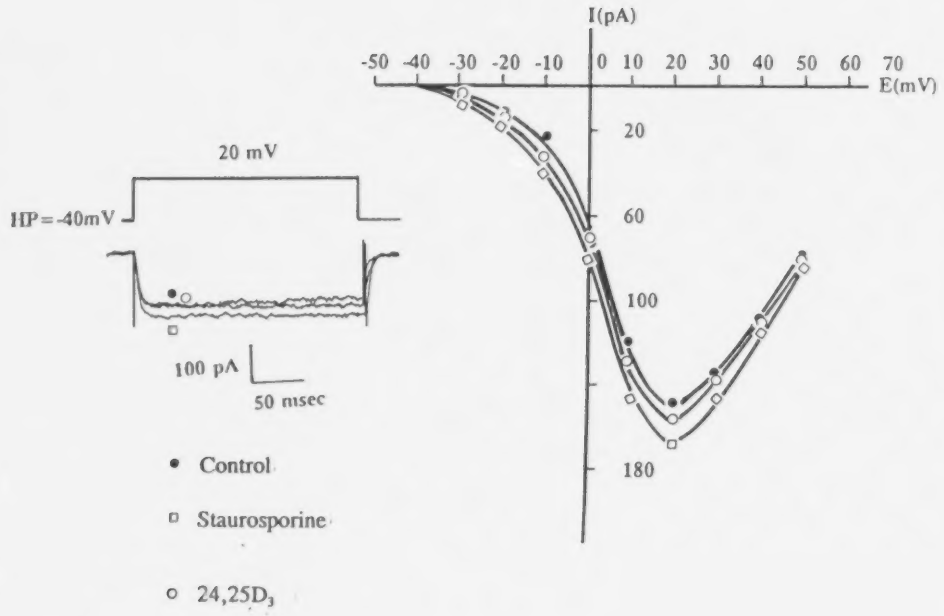

Fig.V-7. The effect of a high concentration of $24,25D_3$ on the L type calcium channel currents in a UMR-106 cell before and after staurosporine. The currents shown at left were activated by depolarizing the cell to 10 mV from a holding potential of -40 mV. The control current (filled circles) was slightly increased from 148 pA to 172 pA 10 min after the addition of staurosporine (open squares). A subsequent addition of $1x10^{-5}$M of $24,25D_3$ had no further effect on the current amplitude (open circles).

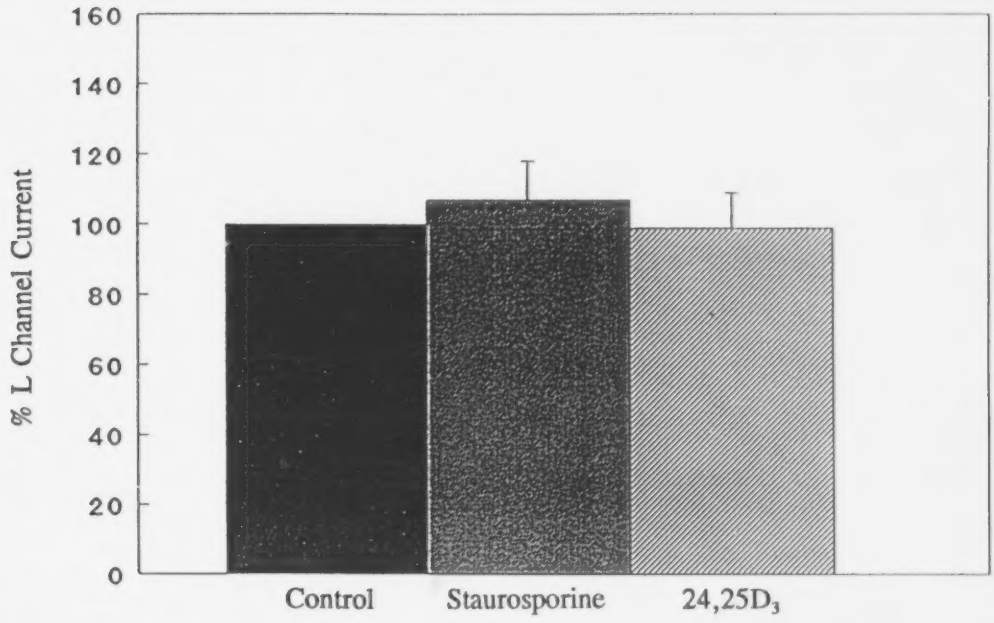

Fig. V-8. The effect of $24,25D_3$ on the L type calcium channel currents in UMR-106 cells before and after staurosporine. Staurosporine ($1x10^{-7}M$) slightly but not significantly increased the peak current amplitudes by $7\pm13\%$ ($n=7$, $p>0.05$). The subsequent addition of $1x10^{-5}M$ of $24,25D_3$ did not cause a significant decrease in the current amplitudes.

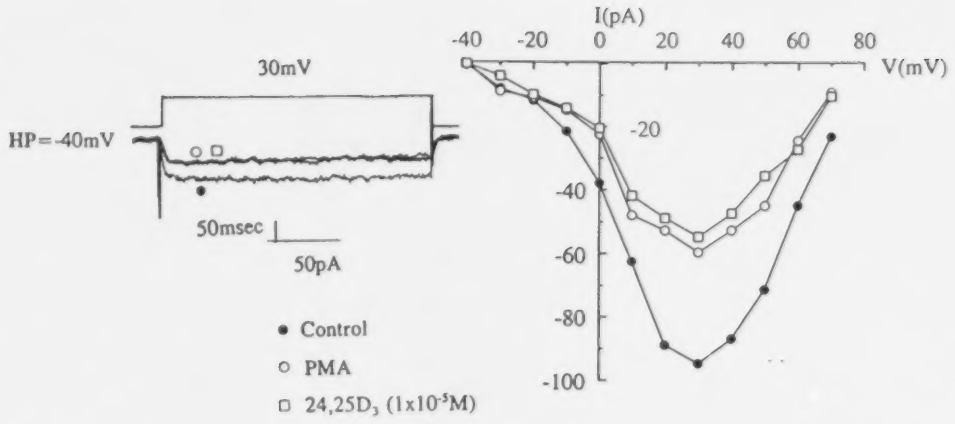

Fig. V-9. The effects of PMA and $24,25D_3$ on the L type calcium channel current in one UMR-106 cell. PMA (open circles) at a concentration of $3x10^{-7}$M decreased the contrrol current amplitude (filled circles) from 97 pA to 58 pA. The subsquent application of $1x10^{-5}$M of $24,25D_3$ (opened squares) did not cause a further decrease in the current amplitude. The corresponding I-V curves are presented on the right side of the figure.

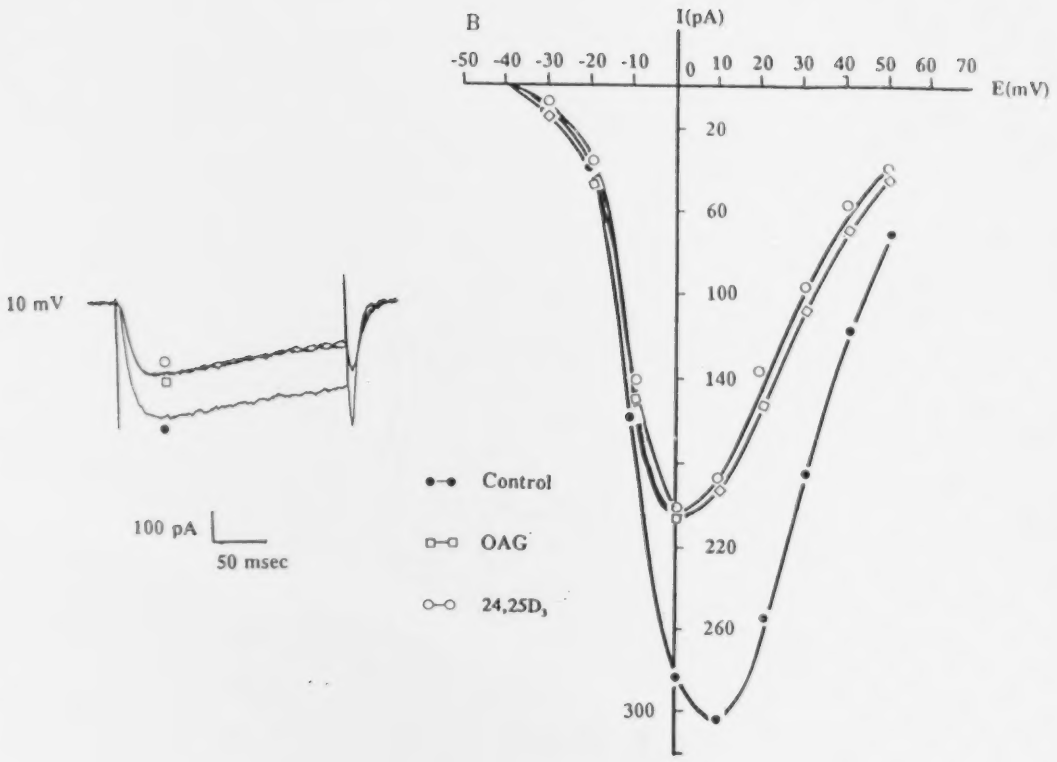

Fig. V-10. The effect of a high concentration $24,25D_3$ ($1x10^{-5}M$) on the L type calcium channel current in a UMR-106 cell after OAG ($1x10^{-5}M$). The currents shown at the left were activated by depolarizing the cell to 10 mV from a holding potential of -40 mV. After the control current (filled circles) was obtained, the cell was treated with OAG $1x10^{-5}M$ for 10 min (open squares) and then stimulated by $24,25D_3$ $1x10^{-5}M$ (open circles). OAG decreased the current amplitude from 306 pA to 204 pA and shifted the peak of the I-V curve from -10 mV to 0 mV. The subsequent addition of $24,25D_3$ had no further effect on the current.

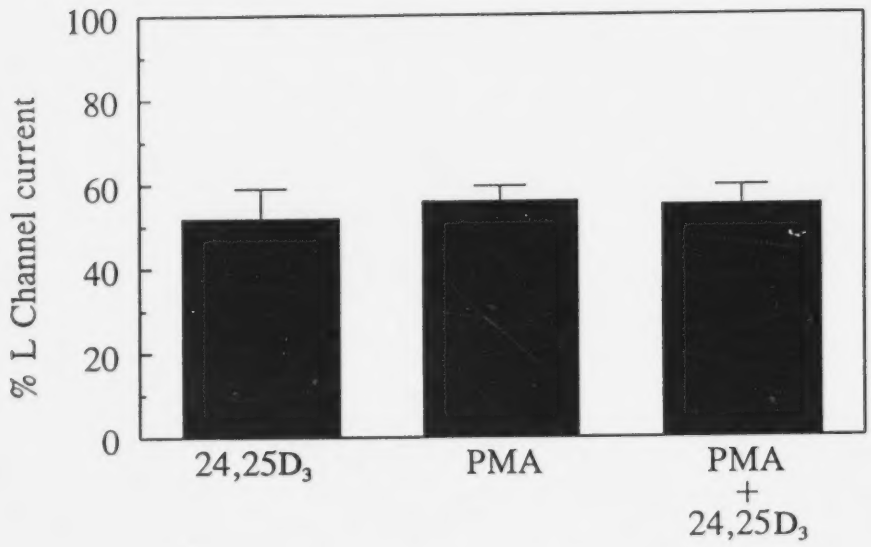

Fig. V-11. The summary of the effects of PMA and $24,25D_3$ on the L type calcium channel currents in UMR-106 cells. $24,25D_3$ at a concentration of $1x10^{-5}M$ decreased the current amplitude by $49\pm7\%$ ($n=9$, $p<0.05$). PMA at a concentration of $3x10^{-7}M$ decreased the current amplitude by $43\pm4\%$ ($n=5$, $p<0.05$). The subsequent application of $1x10^{-5}M$ of $24,25D_3$ did not cause a significant decrease in the current amplitude ($n=5$, $p>0.05$).

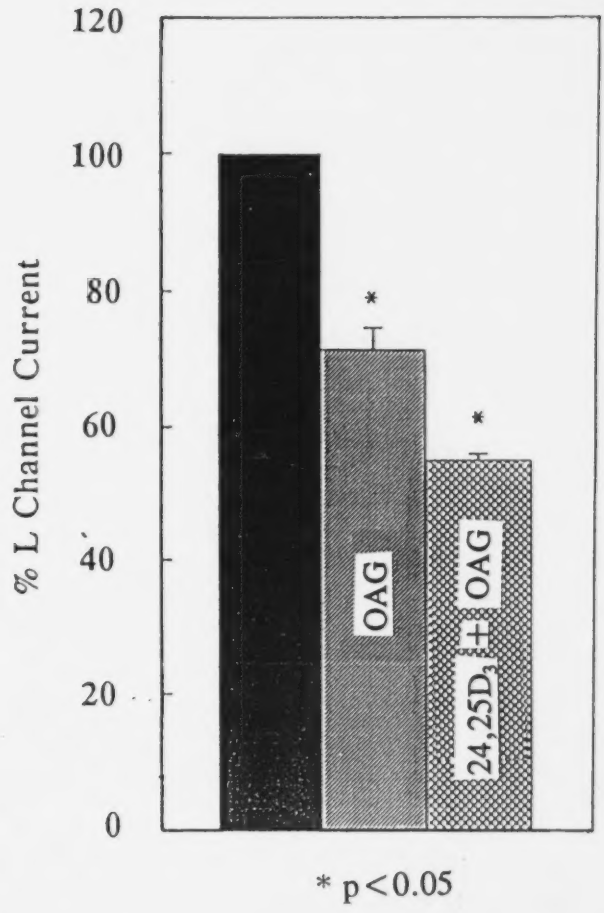

Fig. V-12.The effects of one concentration of $24,25D_3$ and OAG on the L type calcium current in UMR-106 cells. In a group of 5 cells, OAG ($1x10^{-5}$M) treatment decreased the peak current amplitudes by $29\pm4\%$. The $24,25D_3$ ($1x10^{-5}$M) further decreased the current peak amplitude by $44\pm4\%$ ($n=5$, $p<0.05$).

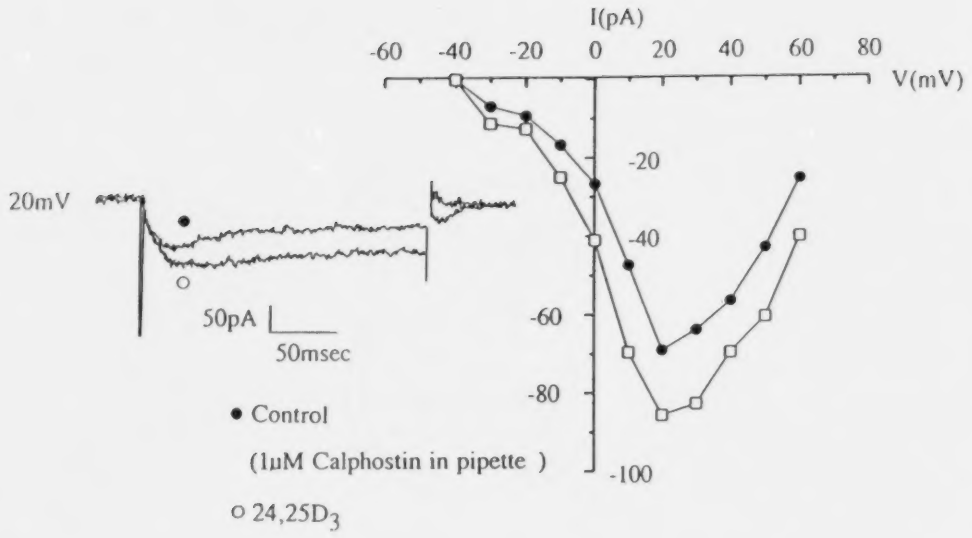

Fig. V-13. The effect of $24,25D_3$ on the L type calcium channel current in the presence of intracellular calphostin C in a UMR-106 cell. The currents were activated by depolarizing the cell to 20 mV from a holding potential of -40 mV. Calphostin C ($1x10^{-6}$ M) was added in the pipette solution. The control currents were recorded after the cell was perfused with calphostin C for 10 min. Subsequent application of $24,25D_3$ $1x10^{-7}$M increased the current amplitude from 68 pA to 86 pA. The I-V relationships are shown on the right.

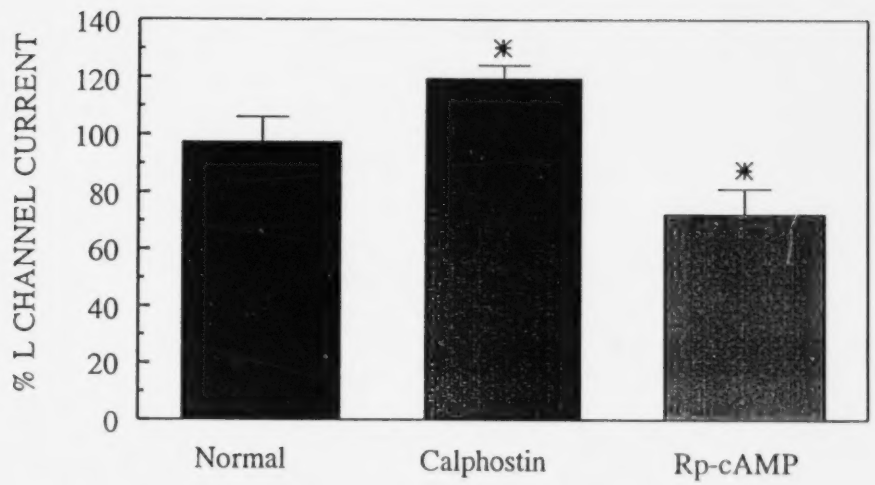

Fig. V-14. The effects of $24,25D_3$ ($1x10^{-7}M$) on the L type calcium channel currents with or without Rp-cAMPs or calphostin C in the pipette solution. With the normal pipette solution (without Rp-cAMPs or calphostin C), $24,25D_3$ ($1x10^{-7}M$) had no effect on the current. With calphostin C ($1x10^{-6}M$) in the pipette solution, $24,25D_3$ ($1x10^{-7}M$) increased the current amplitude by $19\pm4\%$ (n=4, *, $P<0.05$). In contrast, with Rp-cAMPs ($1x10^{-7}M$) in the pipette solution, the same concentration of $24,25D_3$ decreased the current amplitude by $28\pm9\%$ (n=4, *, $P<0.05$).

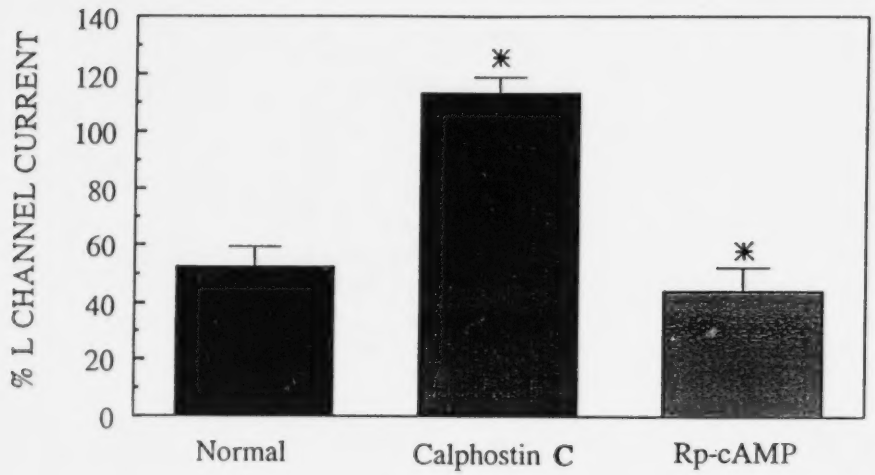

Fig. V-15. The effects of $24,25D_3$ ($1x10^{-5}M$) on the L type calcium channel currents with or without Rp-cAMPs or calphostin C in the pipette solution. With the normal pipette solution (without Rp-cAMPs or calphostin C), $24,25D_3$ $1x10^{-5}M$ decreased the peak current amplitude by $43\pm6\%$ ($n=11$, *, $p<0.05$). With calphostin C ($1x10^{-6}M$) in the pipette solution, $24,25D_3$ ($1x10^{-5}M$) increased the current amplitude by $12\pm5\%$ ($n=4$, *, $p<0.05$). In contrast, with Rp-cAMPs ($1x10^{-7}M$) in the pipette solution, the same concentration of $24,25D_3$ decreased the peak current amplitude by $56\pm4\%$ ($n=4$, *, $p<0.05$).

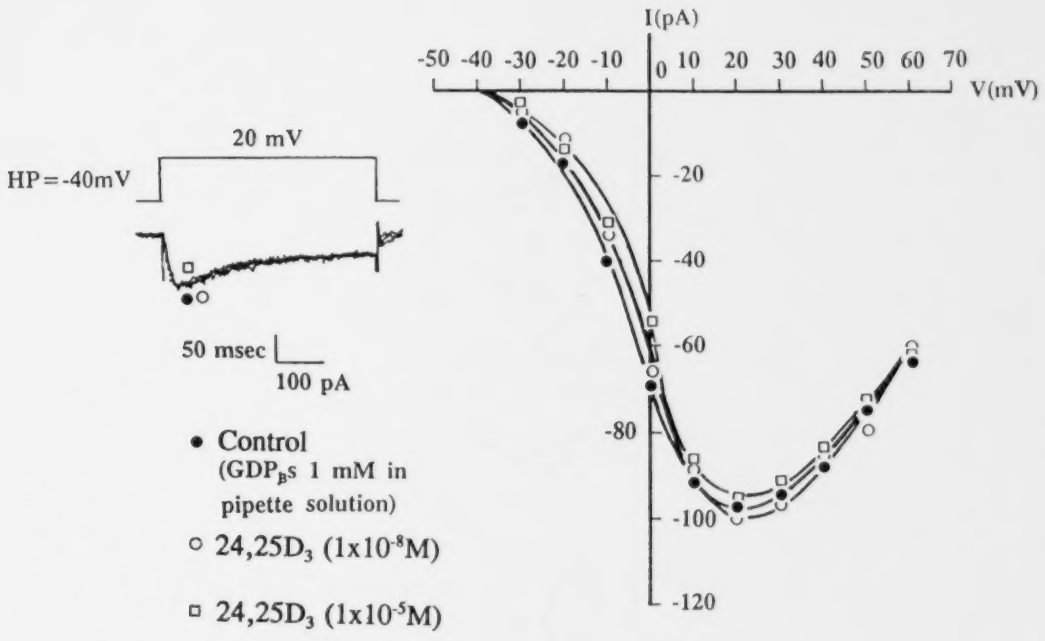

Fig. V-16. The effects of two concentrations of $24,25D_3$ on the L type calcium channel currents in a UMR-106 cell perfused with GDPβs. In this experiment, the pipette solution contained $1x10^{-3}$M of GDPβs. The currents shown at left were activated by depolarizing the cell membrane to 20 mV from a holding potential of -40 mV. The control current was recorded after the cell was perfused with GDPβs for 10 min. The subsquent addition of a low ($1x10^{-8}$M) and a high ($1x10^{-5}$M) concentrations of $24,25D_3$ had no further effects on the L type calcium current amplitudes. The I-V curves shown on the right were not changed by the application of $24,25D_3$ with GDPβs in the pipette solution.

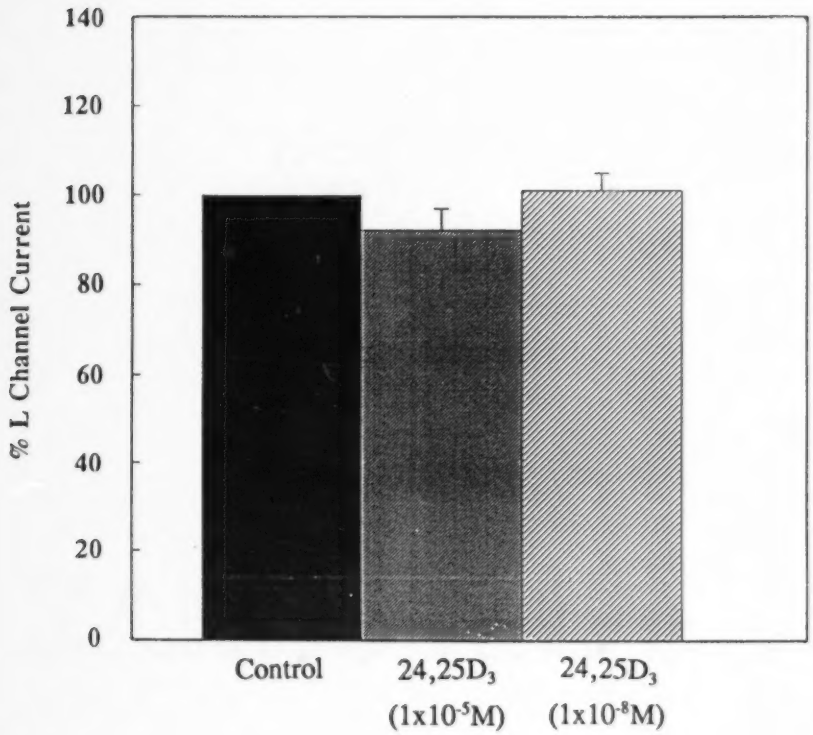

Fig. V-17. The effects of $24,25D_3$ on the L type calcium channel currents in cells perfused with GDPβs. With $1x10^{-3}$M GDPβs in the pipette solution, the enhancing effect of $1x10^{-8}$M $24,25D_3$ and the inhibitory effect of $1x10^{-5}$M $24,25D_3$ on the L type calcium channel currents were abolished.

5.4. Discussion

There is a large body of evidence showing that calcium channels are modulated by intracellular second messenger systems (Rane and Dunlap, 1986; Shearman *et al.*, 1989; Vivaudou *et al.*, 1988; Ishikawa *et al.*, 1993). Molecular biological studies have revealed important information about the primary sequence of voltage-dependent calcium channel proteins. It has been demonstrated that the sequence of calcium channel proteins has several sites which can be phosphorylated by a family of protein kinases such as PKC and PKA (Spedding, 1987; Shearman *et al.*, 1989). Once the calcium channel protein is phosphorylated, the gating of the channel is affected. The effect of each kinase on the calcium channel may result in an increase or decrease in the current (Shearman *et al.*, 1989).

The phosphorylation of the channel protein is one of the major mechanisms by which hormones and neurotransmitters modulate calcium channel currents (Spedding 1987). In Chapter IV, it was demonstrated that 24,25D_3 has a biphasic effect on the L type calcium channel in UMR-106 cells. The fact that different concentrations of 24,25D_3 result in opposite actions on the same target cell suggests the involvement of multiple signal systems. As described in the introduction of this chapter, 24,25D_3 appears to activate multiple signal pathways in bone cells, and it is possible that the biphasic effect of 24,25D_3 on the calcium channel is mediated by multiple signal pathways. This hypothesis was examined and it was concluded that both PKA and PKC were involved in the biphasic effect of 24,25D_3 on the L type calcium channels in UMR-106 cells.

5.4.1. Involvement of the cAMP/PKA pathway in the action of $24,25D_3$ on the L type calcium channel current in UMR-106 cells

The effect of cAMP/PKA on the L type calcium channel has been shown to be cell-type dependent. Depending on the cell type, activation of cAMP/PKA may cause inhibition or potentiation of this current. For instance, intracellular application of cAMP directly enhanced the Ca^{2+} current in rat dorsal root ganglia neurons (Fedulova *et al.*, 1985). In cardiac myocytes, the Ca^{2+} current is increased by activation of cAMP-dependent phosphorylation of the calcium channel (Osterrieder *et al.*, 1982; Wahler *et al.*, 1990). In contrast, in smooth muscle cells from rat tail artery, dibutyryl-cAMP inhibited the L type calcium current (Wang *et al.*, 1990). In UMR-106 cells, PTH has been shown to be a strong stimulator of cAMP and to increase calcium influx and intracellular calcium in a cAMP-dependent manner (Yamaguchi *et al.*, 1987; Reid *et al.*, 1987; Kumagai *et al.*, 1989). These observations indirectly suggest that cAMP may modulate calcium channels in bone cells.

Consistent with these observations, our data demonstrate that activation of the cAMP/PKA signal pathway results in an increase in the L type calcium current and the effect of low concentration of $24,25D_3$ on the L type calcium channel in UMR-106 cells is mediated by the cAMP/PKA signal pathway. The evidence which supports this conclusion is as follows:

1) The 8-bromo-cAMP and a low concentration of $24,25D_3$ had a similar, though not totally identical, effect on the L type calcium channel in UMR-106 cells. The 8-

bromo-cAMP concentration-dependently increased the L type calcium current in UMR-106 cells, suggesting that activation of the cAMP/PKA signal pathway results in an increase in the amplitude of the L type calcium channel current.

2) Rp-cAMPs, a PKA inhibitor, when applied intracellularly, blocked the effect on the L type calcium current induced by a low concentration of $24,25D_3$, indicating that the increase in the L type calcium current caused by $24,25D_3$ is cAMP/PKA dependent.

3) When the current was maximally increased by 8-bromo-cAMP, subsequent addition of a low concentration of $24,25D_3$ did not induce a further increase in the current amplitude, indicating that the effect of 8-bromo-cAMP and a low concentration $24,25D_3$ on the L channel current was not additive. A non-additive effect is a common phenomenon when two agents use an identical pathway (Kameyama *et al.*, 1985). This strongly suggests that 8-bromo-cAMP and a low concentration of $24,25D_3$ likely utilize an identical mechanism in increasing the L type calcium current. Therefore, at low concentrations, $24,25D_3$ likely activates the cAMP/PKA signal pathway, and the resultant phosphorylation of the L type calcium channel proteins causes a change in channel gating, which leads to an increase in current amplitude.

However, $24,25D_3$ did not cause a significant increase in the accumulation of cAMP in a monolayer culture of UMR-106 cells. This observation is against a direct activation of the cAMP/PKA pathway by $24,25D_3$. Activation of PKA depends on elevation of intracellular cAMP, and coupling of cAMP to the binding site on the regulatory subunit of PKA (Gwosdow *et al.*, 1994). This results in the dissociation of the

catalytic subunits from the regulatory subunits and leads to the activation of PKA. If $24,25D_3$ does not cause an increase in intracellular cAMP, can the effect of $24,25D_3$ on the L type calcium channel current still be mediated cAMP/PKA pathway? There are several possible explanations:

1) Although cAMP accumulation was not increased by $24,25D_3$, in selected experiments, cAMP could be detected in the medium (data not shown). Whether this is a reflection of a transient increase in the release of cAMP caused by $24,25D_3$ remains unclear. It is possible that $24,25D_3$ may cause a small increase in the intracellular cAMP concentration and the subsequent release of cAMP into the medium without a detectable increase in cAMP accumulation. A small change in intracellular cAMP induced by $24,25D_3$, if transient, could be hard to detect. However, a small increase in cAMP could be enough to activate PKA in UMR-106 cells.

2) Another possible explanation is that the L channel current in UMR-106 cells is highly sensitive to the regulation by the cAMP/PKA pathway. Unlike other cell types that require up to the mM range of 8-bromo-cAMP to saturate the effect on the current, the maximal effect in UMR-106 cells was observed with μM concentration of 8-bromo-cAMP ($5x10^{-5}$M). Thus, a small increase in cAMP accumulation could activate the cAMP/PKA pathway. Another possibility is that $24,25D_3$ may stimulate PKA directly without the involvement of cAMP as previously reported in the effect of IL-1a on AtL-20 cells (Gwosdow *et al.*, 1990; 1993; 1994). In AtL-20 cells, IL-1a increased PKA activity by a cAMP-independent mechanism. Two possibilities were proposed. It was suggested that IL-1a either caused a small but undetectable increase in cAMP or IL-1a had a direct

effect on PKA. The results in this thesis appear to support the first speculations.

3) A third possibility is that treatment with a low concentration of $24,25D_3$ may cause a localized increase in cAMP (compartmentalization of cAMP). It is possible that a localized increase in cAMP in the cytosol may lead to activation of PKA. This is supported by a recent study by Marx and Barsony (1994). They found that interaction of vitamin D_3 agonists with the vitamin D receptors led to the following sequential changes in vitamin D receptors: clumping, centripetal alignment along microtubules, movement to the nuclear membrane, intranuclear uptake, and intranuclear aggregation. They also reported cGMP accumulation near the clumped vitamin D receptors at each step during this reorganization process (Marx and Barsony, 1994). It is possible that similar changes in cAMP may also occur upon the activation of vitamin D receptors without affecting the total concentration of cAMP in the cytosol. Such a localized increase in cAMP around the activated vitamin D receptors may be sufficient to activate PKA. The precise mechanism by which $24,25D_3$ increases the amplitude of the L type calcium current via PKA remains to be determined. Additional studies on the time-course of cAMP accumulation induced by $24,25D_3$ and the application of other specific PKA inhibitors on the effect of $24,25D_3$ on the L type calcium channel may reveal valuable information.

5.4.2. Involvement of the PKC pathway on the action of $24,25D_3$ on the L type calcium channel current in UMR-106 cells

There is evidence that PKC modulates the activity of different ion channels in

many cell types (Shearman *et al.*, 1989). In bone cells, vitamin D_3 metabolites have been demonstrated to activate the PLC/PKC signal pathway (Yamaguchi *et al.*, 1989). Specifically, $24,25D_3$ has been shown to increase IP_3 and DAG in osteoblasts (Grosse *et al.*, 1993). Furthermore, some effects of bone-modelling hormones and steroids on intracellular calcium have also been linked to PKC activation (Bos *et al.*, 1992). For example, pretreatment of osteosarcoma cells with PTH abolished the phorbol ester-induced increase in free intracellular calcium (Yamaguchi *et al.*, 1987). The increase in the L type calcium channel activity induced by $1,25D_3$ in skeletal muscle cells and osteosarcoma cells have been shown to be modulated by PKC (Yamaguchi *et al.*, 1987; de Boland *et al.*, 1993), suggesting the involvement of PKC in the effect of vitamin D_3 on the L type calcium channels. However, little is known about the role played by PKC on the effect of $24,25D_3$ on the L type calcium channel.

The data presented in this study demonstrate that PKC mediates the inhibitory effect of $24,25D_3$ on the L type calcium channel current in UMR-106 cells. The following evidence supports this conclusion.

1) Activation of PKC by PMA and OAG results in inhibition of the L type calcium channel current similar to that of a high concentration of $24,25D_3$. The PMA and OAG-induced inhibition of the L type calcium channel current suggests that activation of PKC may mediate the inhibition of the L type calcium current by $24,25D_3$.

2) The inhibition of the L type calcium current caused by PMA and a high concentration of $24,25D_3$ were not additive. When added alone, PMA ($3x10^{-7}M$) and

$24,25D_3$ ($1x10^{-5}M$) decreased the current by 43±3% and by 49±7%, respectively. Addition of $24,25D_3$ after treatment with a maximal concentration of PMA, did not cause a further inhibition of the L type calcium current. This observation suggests that PMA and high concentration of $24,25D_3$ likely activate an identical pathway, *i.e*, PKC.

3) Pretreatment of the cells with a PKC specific inhibitor, calphostin C and a nonspecific protein kinase inhibitor, staurosporine, blocked the inhibitory effect of $24,25D_3$ on the L type calcium current. This confirms the observations from experiments using PKC activators. Based on these observations, it is concluded that the inhibitory effect of $24,25D_3$ is mediated by the PKC pathway.

However, the inhibition of the current amplitude caused by OAG ($1x10^{-5}M$) was not as potent as that of PMA ($3x10^{-7}M$) and the subsequent addition of $1x10^{-5}M$ of $24,25D_3$ could cause a further inhibition on the current amplitude. This may reflect that the concentration of OAG used in these experiments may be a submaximal concentration which only partly activates the PKC pathway. Therefore, the addition of $24,25D_3$ results in a further activation of PKC which caused a further decrease in the current.

5.4.3. The time course of the effect of PKC on the L type calcium channel current

The action of PKC on voltage-dependent calcium channels has been proposed to be dependent on the duration of treatment and to be cell-specific (Shearman *et al.*, 1989). For example, short-term exposure (5 s) of cardiac ventricular cells to TPA resulted in an enhanced L type calcium current whereas longer exposure (20 min) was required to

reduce the current (Lacerda *et al.*, 1988). PMA, but not OAG, has also been reported to have a time-dependent effect on the L type calcium current in rat ventricular cells (Liu *et al.*, 1992).

In UMR-106 cells, PMA and OAG did not exhibit this time-dependent effect on the L type calcium channel currents. The inhibitory effect of PMA and OAG on the L type calcium channel current developed slowly and was sustained for up to 30 min. The sustained action of PMA and OAG may be one of the reasons which accounts for the slow-developing inhibitory effect of $24,25D_3$ on the L type calcium channel. The mechanism of this persistent inhibition by PMA and OAG on the L type calcium channel is not clear. It has been speculated to be a result of a phospholipid-induced change in enzyme conformation (Alkon *et al.*, 1988). Another explanation is that the transferred phosphate moiety is stable, either due to its inaccessibility, or to a concomitant inhibitory action of PKC on the phosphoprotein phosphatase (Ase *et al.*, 1988; Shearman *et al.*, 1989). However, whether these explanations are applicable to the persistent effect of PMA and OAG on the L type calcium channel in UMR-106 cells remains unclear and needs further investigation.

The involvement of PKC in the effect of $24,25D_3$ on the L type calcium current in UMR-106 cells is an important observation. PKC has been linked to some actions of vitamin D_3 and its metabolites in bone cells. For example, activation of PKC has been shown to inhibit $^{45}Ca^{2+}$ accumulation in cultures of osteoblast-like cells (Kozawa *et al.*, 1992). The bone resorption induced by PTH, $1,25D_3$ and prostaglandin E_2 was inhibited

by a specific inhibitor of PKC, AMG (Bos *et al.*, 1992). The involvement of PKC in the action of $24,25D_3$ on calcium channels may account for the action of PKC in bone remodelling.

5.4.4. Involvement of G proteins in the effect of $24,25D_3$ on the L type calcium channel current in UMR-106 cells

G proteins comprise a family of membrane-bound proteins with many subtypes (Birnbaumer,1990). They play a central role as transducers connecting the stimulator to the effector (Simon *et al.*, 1991). Functionally, G_s is the stimulatory G protein that couples the receptor to the catalytic subunit of adenylyl cyclase which leads to the activation of the cAMP/PKA system. G_q is the pertussis toxin- and cholera toxin-insensitive G protein for PIP_2 hydrolysis which leads to activation of the PLC/PKC system (Smracka *et al.*, 1991). Even though the modulation of calcium channels in many cells by most hormones, neurontransmitters and drugs has been shown to be G protein-dependent, it was not known whether or not the effects of vitamin D_3 and its metabolites on the calcium channel in bone cells are mediated by G proteins.

In this study, it was found that the biphasic effect of $24,25D_3$ on the L type calcium channel in UMR-106 cell was blocked by the intracellular application of GDPβs. This strongly suggests that G proteins mediate the action of $24,25D_3$ on the L type calcium channel. This result also provides indirect evidence to support the speculation that a membrane receptor (or binding site) for $24,25D_3$ is coupled to a G protein. Studies with pertussis toxin have suggested that G proteins play an important role in the control

of osteoblast function (Babich *et al.*, 1989). For instance, G proteins are involved in the regulation of alkaline phosphatase activity in ROS 17/2.8 cells (Strewler *et al.*, 1987), and the bone resorption by diverse factors, such as PTH and calcitonin (Rodan and Rodan, 1986; Vargas *et al.*, 1989; Ransjo and Lerner, 1987; 1990). As discussed in the previous chapter, vitamin D_3 and its metabolites (including $24,25D_3$) also modulate these functions. Thus, the mediation of the effects of $24,25D_3$ on the L type calcium channel by G proteins may also be part of the mechanism linked to these physiological functions of $24,25D_3$ on bone.

G proteins can modulate calcium channels either directly or indirectly through second messengers (Sternweis and Pang, 1990). Yatani *et al.* (1987, 1988) demonstrated that purified G_s can directly activate calcium channels incorporated into lipid bilayers where second messenger mechanisms are not present. Alternatively, G proteins can modulate calcium channels indirectly through second messengers. When a receptor is stimulated by a ligand, the associated G proteins lead to the activation of second messenger systems which can, in turn, modulate calcium channels. For example, β-adrenergic agonists have been shown to increase calcium channel currents through G_s and intracellular cAMP-dependent phosphorylation (Nargeot *et al.*, 1983). In the present study, the involvement of G protein is confirmed by studies with the intracellular application of GDPβs. However, it is not clear whether the G proteins activated by $24,25D_3$ act directly or indirectly on the L type calcium channel in UMR-106 cells. Since cAMP/PKA and PKC (the two signalling pathways involved in the dual effects of

$24,25D_3$ on the L type calcium channel) are coupled to G proteins, it is likely that the mediation of the L type calcium channel by G proteins are indirectly through second messengers. In support of this hypothesis, G proteins have been found to activate a phosphoinositide-specific phospholipase C in UMR-106 cells (Babich *et al.*, 1989) and mediate the adenylyl cyclase in ROS 17/2.8 cell (Pines *et al.*, 1986). However, a direct action of G proteins on the L type calcium channel cannot be entirely ruled out.

Since GDPβs is a nonspecific G protein inhibitor, its blocking action on the dual effect of $24,25D_3$ on the L type calcium channel only indicates the involvement of G proteins. The specific types of G proteins involved in these effects of $24,25D_3$ on the L type calcium channel still remain unknown. Further experiments using specific G protein blockers, such as pertussis toxin and cholera toxin or specific G protein antibodies, may define the specific G proteins that are linked to the biphasic effect of $24,25D_3$ on the L type calcium channels in UMR-106 cells.

Chapter VI

General discussion and summary

6.1. General discussion

6.1.1. Voltage-dependent calcium channels in bone cells and their modulation

Intracellular calcium concentration is important for the control of many essential cellular responses. Changes in $[Ca^{2+}]_i$ control cellular functions such as excitability, contraction, or exocytosis and also regulate universal cellular activities, such as metabolism and gene expression (Tsien and Tsien, 1990). Changes in $[Ca^{2+}]_i$ can be achieved by regulating calcium entry via calcium channels, or calcium release from intracellular calcium stores. Voltage-dependent calcium channels play an important role in calcium entry and have been extensively studied in many cells (Spedding and Paoletti, 1992). However, the investigation of voltage-dependent calcium channels has concentrated on excitable cells, such as cardiac myocytes, smooth muscle cells, endocrine cells and neurons. Voltage-dependent calcium channels in these cells provide a major pathway for calcium entry and cause the contraction of cardiac and smooth muscle, neurotransmitter release and the secretion from endocrine cells. In the late 1980's, various studies demonstrated that voltage-dependent calcium channels are also expressed in many nonexcitable cells including bone cells. Based on electrophysiological and

biochemical studies, both the T and L types of calcium channels are expressed in primary cultured bone cells (Chesnoy-Marchais and Fritsch, 1988) and osteosarcoma cell lines (Guggino *et al.*, 1988; Grygorczyk *et al.*, 1989; Karpinski *et al.*, 1989; Morain *et al.*, 1992). The electrophysiological and pharmacological properties of these voltage-dependent calcium channels in bone cells appear to be similar to those in excitable cells (Tsien and Tsien, 1990). The L type calcium channel is sensitive to dihydropyridines and is activated by strong depolarizations while the T channel is not sensitive to dihydropyridines and needs a small depolarization for activation.

Despite these preliminary characterizations, many important properties of voltage-dependent calcium channels in bone cells remain unknown or controversial. For example, the single channel conductance has only been studied in one cell type (ROS 17/2.8) (Caffrey and Farach-Carson, 1989). The current density has never been investigated and the expression of T channels has remained controversial (Caffrey and Farach-Carson, 1989). The activation potential threshold and the peak current of the I-V curve are also different (Chesnoy-Marchais and Fritsch,1989; Duncan and Misler, 1989; Morain *et al.*, 1992). Furthermore, the direct modulation of the L type calcium current by intracellular second messenger systems in bone cells has not been studied. Therefore, a detailed characterization of the electrophysiological properties of these channels and their regulation should provide important information regarding the role of these channels in bone physiology.

This study using UMR-106 cells characterizes the current densities of both L and T channels and demonstrates that the current densities are dependent on the duration of

subculture. This is an important observation since the current density is a parameter indicating the number of channels expressed and is related to the cell cycle. The fact that the decline in current density of the T channel is coupled with an increase in the density of the L channel suggests that these channels likely have different roles in growth and differentiation in UMR-106 cells. In some cell types, the expression of T and L channels have been shown to change as the culture time is prolonged and they appear to regulate the cell function. In smooth muscle cells from the rat tail artery, the expression of both T and L channels decreases as the culture time is prolonged. This change in channel expression appears to be related to the change of cell cycle from the contractile state to the differentiated state (Wang *et al.*, 1989). In neurons from dorsal root ganglion, the expression of L channel has been shown to be related to the release of substance P (Rane *et al.*, 1987). However, the expression of L and T channels in UMR-106 cells is different. It may reflect a different role played by the T channels in UMR-106 cells.

The high density of T channels at the early stage of subculture and lower density as the subculture time is prolonged suggest that this channel may be of importance in the initial differentiation of bone cells. This can be explained by the electrophysiological property of the T channel. It has been reported that the membrane potential in bone cells is between -35 mV to -25 mV (Ferrier *et al.*, 1989), which is the voltage range of activation of the T channel. Some bone modelling hormones (PTH and calcitonin) have been shown to depolarize bone cells (Ferrier *et al.*, 1989). Therefore, when the bone cell membrane is depolarized by these agents, the T channels can be activated. Thus, the T channel may play a role in converting an outside signal to trigger cell differentiation.

The continuous expression of the L channel suggests that the L channel is of importance in the maintenance of differentiation. Since the L channel is the main target for many hormones and steroids, it may also play an important role in the modulation of the physiological function of bone cells. In support of this suggestion, bone resorption has been shown to be affected by Bay K 8644, a specific agonist of the L type calcium channel, and by nifedipine, a specific antagonist of L type calcium channel (Guggino *et al.*, 1989).

As described in chapter III, T channels are mainly expressed in cells from the higher passages. This observation may explain the variation in the observations regarding the expression of the T channel in different studies (Duncan and Misler,1989; Karpinski *et al.*, 1989; Morain *et al.*, 1992). It is possible that if the experiment is performed on cells at low passages, only the L type calcium channel is measured. However, if the experiment is performed on cells from high passages, both T and L type calcium channels are measured. Therefore, when the data describing channel expression is compared, the passage should be taken into consideration.

Whether the increased expression of T channels from the higher passages is a natural development or is due to the culture condition remains unclear. If it is a natural development of the T channel in bone cells, it may be possible that the T channel may play a role in age-related bone disorders. However, this and other proposed functions of the T channel in bone are only speculations at present and require further investigation.

Our data show that a relatively satisfactory separation between the T and the L type calcium channel currents may be achieved by using a holding potential of -80 and -40 mV since most of the T channel is inactivated at a membrane potential of -40 mV. This explains the variation in the I-V relationships of the L type calcium channels reported by different groups. Since -60 mV or -80 mV was often used as holding potential to activate the L type channel in those experiments, it is possible that both the T and L type calcium channel are activated and this results in the shift of the I-V curves. A good separation of the two channels is important for the evaluation of the effect of hormones and steroids on the L type calcium channels in UMR-106 cells.

6.1.2. The nongenomic effect of $24,25D_3$ on the L type calcium channel current

The nongenomic effects of vitamin D_3 and its metabolites, as well as other steroids, are currently receiving more and more attention. It has been shown that most of the steroids have nongenomic effects. Some of these effects are, for example, 17β-estradiol on $[Ca^{2+}]_i$ in granulosa cells (Morly *et al.*, 1992), progesterone on $[Ca^{2+}]_i$ in oocytes (Wassermann *et al.*, 1980), aldosterone on the Na^+/H^+ antiport in rat aorta vascular smooth muscle cells (Christ *et al.*, 1993) and $1,25D_3$ on voltage-dependent calcium channels in ROS 1.7/28 cells and smooth muscle cells from rat tail artery (Caffery, 1989; Shan *et al.*, 1993).

The nongenomic effect of $24,25D_3$ in bone remains unclear and controversial. The $24,25D_3$ has either been shown to have no effect or to cause a rapid and sustained

increase in $[Ca^{2+}]_i$. The present study provides evidence demonstrating that $24,25D_3$ has a nongenomic action on the intracellular second messenger systems and the L type calcium channel. The inhibitory effect of $24,25D_3$ on the L type calcium channel current has also been described in ROS 17/2.8 cells (Caffery, 1989). All genomic effects of steroids, as discussed in chapter I, are characterized by a latency period of 1 h or longer (Wehling, 1994). The effect of $24,25D_3$ on the L type calcium channel and intracellular second messenger systems was seen within 5 min after the application of $24,25D_3$. Therefore, this apparently represents a nongenomic action of $24,25D_3$.

As shown in chapter IV, the effect of a low concentration of $24,25D_3$ was similar to that of $1,25D_3$ at a concentration of $1x10^{-6}$M. However, $1,25D_3$ has only been reported to increase the L type calcium channel current (Vazquez and de Boland, 1993; Shan *et al.*, 1993) and only one concentration was tested in this study. Therefore, it is not clear whether or not $1,25D_3$ has a biphasic effect on the L type calcium channel current. Whether $24,25D_3$ and $1,25D_3$ interact with identical or different binding sites also remains unknown.

6.1.3. The integration of PKA and PKC on the effect of $24,25D_3$ on the L type calcium channel current in UMR-106 cells

Dihydropyridine-sensitive calcium channels are regulated by the phosphorylation of channel proteins (Reuter 1983; Tsien *et al.*, 1986). Molecular biological studies have demonstrated that the L type calcium channels are hetero-oligomeric proteins, with α_1,

α_2, β, ω, δ subunits (Leung *et al.*, 1987, 1988; Vaghy *et al.*, 1987). *In vitro* studies have shown that the α_1 and β subunits are substrates for phosphorylation by protein kinases including PKA and PKC (Curtis *et al.*, 1985; Jahn *et al.*, 1988; Nastainczyk *et al.*, 1987; O'Callahan *et al.*, 1988; Hell *et al.*, 1993). The phosphorylation of these subunits has been shown to modulate channel activities. At the level of the whole cell current, this change in activity of channel results in an increase or decrease in current (Chang *et al.*, 1991).

PKA and PKC have been shown to have their own specific phosphorylation sites on the α_1 and β subunits (Chang *et al.*,1993). There is evidence to indicate that some phosphorylation sites can be phosphorylated by both PKA and PKC (Braha *et al.*, 1993). The phosphorylation of channel proteins by PKA or PKC can produce either augmentation or attenuation of the channel activity depending on the cell type. The integration of the effects of PKA and PKC on the channel will determine the net effect on channel activity (Shearman *et al.*, 1989). It can be an additive effect (either enhancing or inhibitory), if the phosphorylation of the channel protein caused by PKA or PKC induces a similar effect, as in the case of the effect of 5-HT on the L type calcium channel in Aplysia cells (Braha *et al.*, 1993). If the phosphorylation of the channel protein caused by PKA or PKC induces different results, the final effect on the channel activity will be non-additive and may be dependent on the kinase which plays the dominant role.

In this study, it was found that the biphasic effects of $24,25D_3$ on the L type

calcium channel current are mediated by PKA and PKC signal pathways. It appears that the enhancing effect is mainly mediated by PKA and the inhibitory effect involves both PKA and PKC with PKC playing the dominant role. The evidence which supports this conclusion is as follows:

1) When the cAMP/PKA signal pathway was inhibited by the intracellular application of Rp-cAMPs, the enhancing effect of a low concentration $24,25D_3$ on the current was abolished but no reversal occurred, suggesting that at this low concentration, $24,25D_3$ mainly activates the cAMP/PKA but not the PKC system. If the PKC system was also activated by this concentration of $24,25D_3$, once PKA was blocked, an inhibitory effect caused by the activation of PKC should appear.

2) With intracellular Rp-cAMPs, $24,25D_3$ at a concentration of $1x10^{-7}M$, which had no effect on the L type calcium current with a normal pipette solution, decreased the L type calcium current, suggesting that, in addition to activation of PKA, PKC was also activated by $24,25D_3$ at this concentration. It seems reasonable to speculate that the lack of effect of $24,25D_3$ on the L calcium channel current at a concentration of $1x10^{-7}M$ may be due to the simultaneous activation of PKA and PKC by $24,25D_3$ at this concentration. Therefore, if this balance between PKA and PKC is shifted by blocking the PKA, the net effect of $24,25D_3$ on the L calcium current becomes inhibitory as shown in Chapter V.

This hypothesis was further supported as a result of using a higher concentration

of $24,25D_3$. With the inhibition of PKA by Rp-cAMPs, the inhibitory effect of $24,25D_3$ at a concentration of $1x10^{-5}M$ was greater. This suggests that when the concentration of $24,25D_3$ was increased to $1x10^{-5}M$, in addition to the activation of cAMP/PKA system, the PKC pathway is also activated and the net result was a decrease in the L type calcium current.

3) If this speculation is true, one anticipates a reversal of the effect of higher concentrations of $24,25D_3$ once the PKC pathway is blocked. This was confirmed by the experiments using PKC blockers. When the PKC pathway was inhibited by the intracellular application of calphostin C, $24,25D_3$ at a concentration of $1x10^{-7}M$ caused an increase in the L type calcium current. Calphostin C also reversed the inhibitory effect of a high concentration of $24,25D_3$ ($1x10^{-5}M$).

Taken together, it appears that $24,25D_3$ at low concentrations ($<1x10^{-8}M$) increases the L type calcium current through the cAMP/PKA signal pathway whereas, at concentrations higher than $1x10^{-8}M$, both cAMP/PKA and PKC signal pathways are activated. At concentrations higher than $1x10^{-7}M$, the effect of the PKC signal pathway predominates and overrides that of cAMP/PKA pathway resulting in an inhibition of the current. This proposed mechanism of $24,25D_3$ on the L type calcium current is shown in Fig. VI-1.

These studies provide evidence for the involvement of the PKA and PKC

pathways in the effect of $24,25D_3$ on the L type calcium channel current. However, the role played by other second messenger systems in this process remains unknown. Since the level of cGMP has been shown to be increased by $1,25D_3$ in fibroblasts (Marx, 1994), it is possible that cGMP and the cGMP-dependent kinases may also be activated by $24,25D_3$ and modulate voltage-dependent calcium channels in UMR-106 cells. An inhibitory effect of cGMP on the L type calcium channel current has been found in several tissues (Mery *et al.*, 1991; Tohse and Sperelakis, 1991; Ishikawa *et al.*, 1993). To completely evaluate the effect of $24,25D_3$ on the calcium channels in bone cells, the possible involvement of cGMP/PKG on the calcium channel currents also needs to be considered.

6.1.4. Clinical significance

The confirmation of the nongenomic effect of $24,25D_3$ is important. The nongenomic effect of vitamin D_3 and its metabolites on intracellular calcium has been considered as the early action on bone and may play a role in regulating the genomic effect of these steroids. The alteration of intracellular calcium has been linked to bone remodelling induced by some hormones (Martin and Partridge, 1981; Marcus and Orner, 1980; Kumagai *et al.*, 1989), bone resorption (van Leeuwen *et al.*, 1992), and the synthesis of some proteins in bone cells (van Leeuwen *et al.*, 1992). Since $24,25D_3$ has a nongenomic biphasic effect on the L type calcium channels and changes in intracellular calcium concentration, this strongly suggests that $24,25D_3$ has an effect on the function of bone cells. The results in this thesis and other reports on the nongenomic effect of

$24,25D_3$ on the calcium channels and intracellular calcium do not support the conclusion that $24,25D_3$ is biologically inert at physiological concentrations (Civitteli and Avioli, 1994). In contrast, it suggests that $24,25D_3$ may play a role in the modulation of function of bone cells via its action on the voltage-dependent calcium channels.

As shown in chapter IV, low concentrations of $24,25D_3$ and $1,25D_3$ have a similar effects on the L type calcium channel. Since $24,25D_3$ has a much lower affinity than $1,25D_3$ for the vitamin D receptor, the effect of $24,25D_3$ on the calcium channel at physiological concentrations may be over shadowed by that of $1,25D_3$. In some special circumstances, for example, under pharmacological conditions, high concentrations of $24,25D_3$ may have an impact on bone cells. This hypothesis is supported by several observations. High concentrations of $24,25D_3$ have been shown to antagonize some actions of $1,25D_3$ (Kriajev and Edelstein, 1994). A large concentration of $24,25D_3$ has been shown to be effective in the treatment of osteomalacia and has less side effects than $1,25D_3$ (Bordoer *et al.*, 1978). The biphasic effect of $24,25D_3$ on the L type calcium channel may be important in the anabolic effects of $24,25D_3$. However, this speculation remains to be confirmed.

6.2. Summary

6.2.1. Two types of the voltage-dependent calcium channels are identified and characterized in UMR-106 cells. The electrophysiological and pharmacological properties

of these two types of calcium channels are identical to those of L and T type calcium channels.

6.2.2. The pattern of the expression of the two channels is different. The L type calcium channel current can be detected in the cells from all passages and the channel density increases slightly as the culture time is prolonged. In contrast, the T type calcium channel current is mainly expressed in the cells from higher passages and the channel density is decreased as the culture time prolonged. The T type calcium channel has the highest density in the first 10 hours and the lowest density after 48 hours in culture.

6.2.3. The $24,25D_3$ has a biphasic effect on the L type channel current while it has no effect on T channel current. In the low concentration range ($<5x10^{-8}M$). $24,25D_3$ increases the L type calcium channel current. In the high concentration range ($>1x10^{-7}M$), $24,25D_3$ decreases the L type calcium channel current.

6.2.4. The biphasic effect of $24,25D_3$ on the L type calcium channel occurs rapidly and is reversible by washout, suggesting that it is a nongenomic effect and likely acts through a membrane-associated receptor or binding site.

6.2.5. Activation of the cAMP/PKA signal pathway results in an increase in the L type calcium channel current. In contrast, activation of the PKC signal pathway results in a decrease in the L type calcium channel current.

6.2.6. The biphasic effect of $24,25D_3$ on the L type calcium channel is mediated, at least, by the cAMP/PKA and PKC signal pathways. At the low concentration range, $24,25D_3$ activates the cAMP/PKA signal pathway which leads to an increase in the L type calcium channel current. At the high concentration range, $24,25D_3$ activates both the cAMP/PKA and PKC signal pathways and the net result is a decrease in the L type calcium channel current.

6.2.7. G proteins are involved in the biphasic effect of $24,25D_3$ on the L type calcium channel current.

6.3. Significance

6.3.1. This is the first report to show that the L and the T type calcium channels in UMR-106 cells have a different pattern of channel expression and channel density . This may provide a suitable model to study the physiological consequence of a differential expression of T and L type calcium channels.

6.3.2. This study is the first report to demonstrate that $24,25D_3$ modulates the L type calcium channel and this effect depends on the concentration of $24,25D_3$. Furthermore, at least two signalling pathways are involved in the effect of $24,25D_3$. This different effect of $24,25D_3$ may be related to the physiological and pharmacological functions of this steroid hormone.

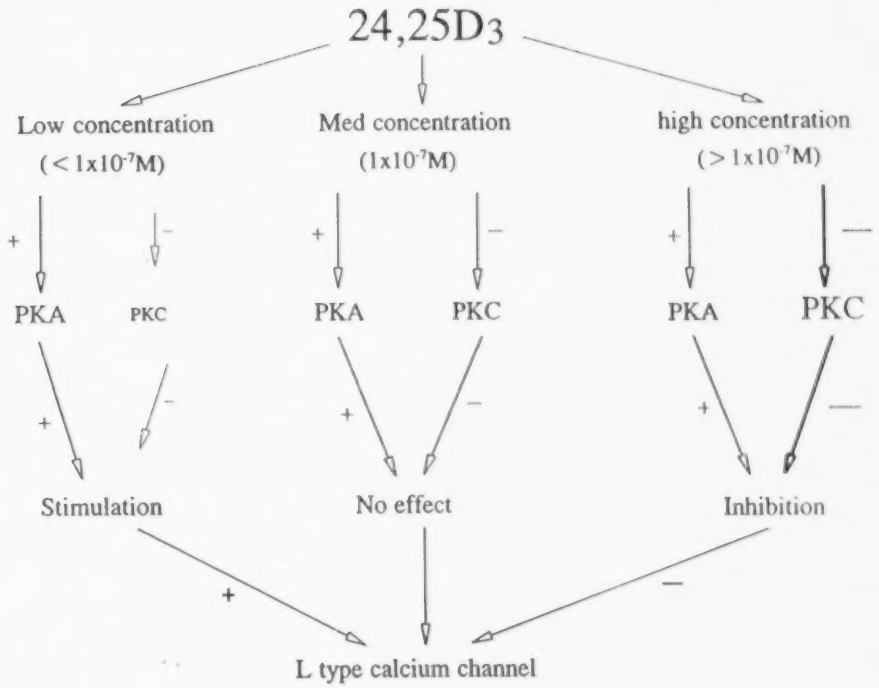

Fig. VI-1. Schematic representation of the proposed mechanism of the effect of $24,25D_3$ on the L type calcium channel current in UMR-106 cells. The different thicknesses of the arrows indicates the potency of the effects of $24,25D_3$ on the second messenger pathways. At concentrations lower than $1x10^{-7}M$, $24,25D_3$ potently activates the PKA pathway which results in an increase in the L type calcium channel current amplitude. At concentrations about $1x10^{-7}M$, $24,25D_3$ probably activats the PKA and PKC signal pathways equally hence causing no effect on the current amplitude. At concentrations higher than $1x10^{-7}M$. the activation of PKC by $24,25D_3$ is more potent than that of PKA resulting in a decrease in the current amplitude.

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